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Curcumin and Hispolon as Potential Antibacterial Agents

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Abstract

Antibiotic resistant bacteria are becoming an increasing threat worldwide, particularly in the healthcare setting. This has led researchers and healthcare providers to begin looking elsewhere for solutions. Research suggests that curcumin, a phenolic compound from the spice turmeric, has antibacterial properties that may be able to treat potentially life-threatening hospital infections, such as those caused by *Staphylococcus aureus* and *Pseudomonas aeruginosa.* Turmeric has been used in Asian medicine for thousands of years as a general antimicrobial. Curcumin was utilized in this study, along with hispolon, another phenolic compound isolated from various mushrooms, such as *Inonotus hispidus* and *Phellinus linteus*, a medicinal mushroom. There is less prior data on hispolon as an antibacterial agent, but it has been found to be a potentially effective antiviral and antitumor treatment. Promising research done so far with hispolon as an antitubercular drug suggests that it may have some antibacterial properties as well. In this study, curcumin, hispolon mono methylether (HME), and hispolon pyrazole (HP) were tested on *Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis, Escherichia coli,* and *Mycobacterium smegmatis*. The results obtained through colony forming unit assays, % inhibition calculations, growth curves, biofilm assays, and live-dead fluorescent microscopy suggest that HME has significant antibacterial effects on all of the microorganisms used except for *E. faecalis*, where the effects are only moderate, while curcumin has moderate antibacterial effects on all but *M. smegmatis.* HP did not have strong antibacterial effects on gram positive or gram negative bacteria, and only seemed to be effective against *M. smegmatis*.

MONTCLAIR STATE UNIVERSITY

Curcumin and Hispolon as Potential Antibacterial Agents

by

Haleigh Sullivan

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1. Introduction

Many kinds of bacteria are beneficial in various aspects of life. Healthy humans tend to have about 10^{14} bacteria just in their gut alone (Zhang *et al.,* 2015). These bacteria play many important roles in health, such as helping with digestion and providing certain nutrients (Zhang *et al.,* 2015). However, not all bacteria are beneficial, and there is a significant number that can cause dangerous illnesses in humans when introduced into the body. Bacterial infections have been a problem for people for as long as humans have existed, and they can be caused by various type of pathogenic bacteria.

Pathogenicity

There are many factors that make certain bacteria pathogenic. Many bacteria produce toxins, such as the lipopolysaccharide endotoxin in gram negative bacteria and teichoic acid in gram positive bacteria (Wilson *et al.*, 2002). Toxins are generally classified into different categories, including A-B toxins, commonly produced by gram negative bacteria such as *Escherichia coli*, and toxins that form pores in host cell membranes (Wilson *et al.,* 2002). Some pathogenic bacteria are enclosed in capsules. These give them extra protection from destruction by macrophages in the host immune system, making it easier for these bacteria to evade host defenses (Wilson *et al.,* 2002). The cell wall can also determine different virulence factors. As previously mentioned, most bacteria are classified as gram negative or gram positive, depending on the peptidoglycan composition in their cell wall (Wilson *et al.,* 2002). Some bacteria also have the ability to break down host tissues in order to invade them. *Staphylococcus aureus* is an example of a pathogenic bacterium that uses this method, and once the tissues are invaded, it is easier for the bacteria to spread to other areas of the body, which then puts the host at risk for sepsis (Wilson *et al.,* 2002). There are many mechanisms that pathogenic bacteria use to evade

the host immune system and successfully cause disease. Therefore, there need to be treatments to either kill these bacteria, or help the immune system eliminate them from the body (Figure 1).

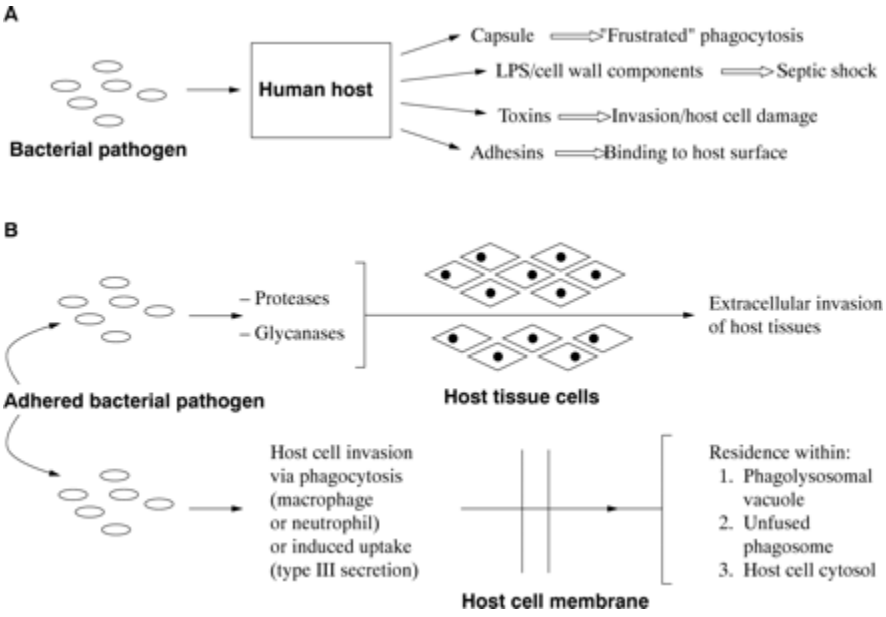

Figure 1. Some features that contribute to pathogenicity in bacteria (Wilson *et al.,* 2002)

Antibacterial Treatments

Since the discovery of penicillin as an antibacterial agent by Alexander Fleming, antibacterial compounds called antibiotics have been an effective treatment for many bacterial infections (Lobanovska & Pilla, 2017). The mode of action for this particular antibiotic is to prevent peptidoglycan formation, which can then potentially result in the bacterial cell bursting (Lobanovska & Pilla, 2017). The discovery of penicillin has led to the development of other antibiotics over time, since different bacteria are susceptible to different ones. For example, gram positive and gram negative bacteria may respond differently to various antibacterial compounds (Lobanovska & Pilla, 2017). Some of these antibiotics include quinolones, which block bacterial DNA replication, aminoglycosides, which inhibit the 30S ribosomal subunit, and macrolides, which prevent protein synthesis (Kapoor *et al.,* 2017). There are currently many different

antibiotics used in modern medicine that have a wide range of actions against pathogenic bacteria (Figure 2). Unfortunately, despite the fact that antibiotics have been in use for less than one hundred years, they are quickly becoming less effective.

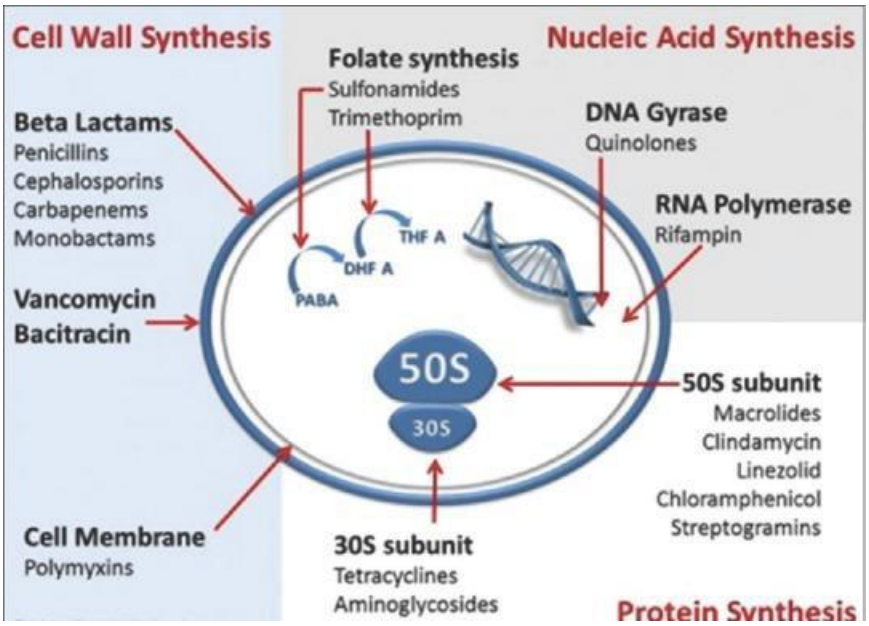

Figure 2. Various modes of action of different antibiotics (Kapoor *et al.*, 2017)

Antibiotic Resistance

Bacteria that are no longer responding to various types of antibiotics are a growing problem in the medical field, leading doctors and scientists to search elsewhere for a solution. Unfortunately, a fairly wide range of bacteria are becoming antibiotic resistant, including those that are both gram positive and gram negative (Tyagi *et al.*, 2015). In hospitals, where these super bug infections are especially a concern, bacteria such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* are becoming an increasing problem (Tyagi *et al.*, 2015). Bacterial infections that can no longer be treated with antibiotics pose a serious threat to future humans. The rise in antibiotic resistance has been attributed mainly to over prescribing antibiotics, as well as people misusing them, such as stopping the medication before instructed by their physician

(Ventola, 2015). Scientists project that by the year 2050, as many as 10 million people each year could die from infections caused by antibiotic resistant bacteria (Banin *et al.,* 2017). This issue has led researchers to begin investigating other potential antibacterial compounds to eventually treat some of these infections.

There are various mechanisms that different bacteria use to become resistant to antibiotics. Gram negative bacteria are generally more resistant to antibacterial agents than gram positive bacteria due to the fact that they have an extra cell wall (Zgurskaya *et al.,* 2015). A fairly common mechanism that leads to antibiotic resistance is horizontal gene transfer (HGT), where one bacterium receives foreign segments of DNA from a different bacterium (Munita & Arias, 2016). Through this, a gene for resistance to certain antibiotics can be passed on to bacteria that did not previously have them. HGT is a major reason for evolution in bacteria as well (Munita & Arias, 2016). Gram negative bacteria do have some mechanisms that are different from gram positive bacteria. Many gram negative bacteria have started producing enzymes known as β-lactamases, which can break down β-lactam rings in certain antibiotics, while a common reason for antibiotic resistance in gram positive bacteria includes them modifying sites where antibiotics would normally bind (Munita & Arias, 2016). In addition to these methods, gram negative and gram positive bacteria both may have efflux pumps, which they can use to quickly pump the antibiotics out of the cell before it can be killed (Munita & Arias, 2016) (Figure 3). Therefore, regardless of gram classification, antibiotic resistance is becoming a very significant issue worldwide.

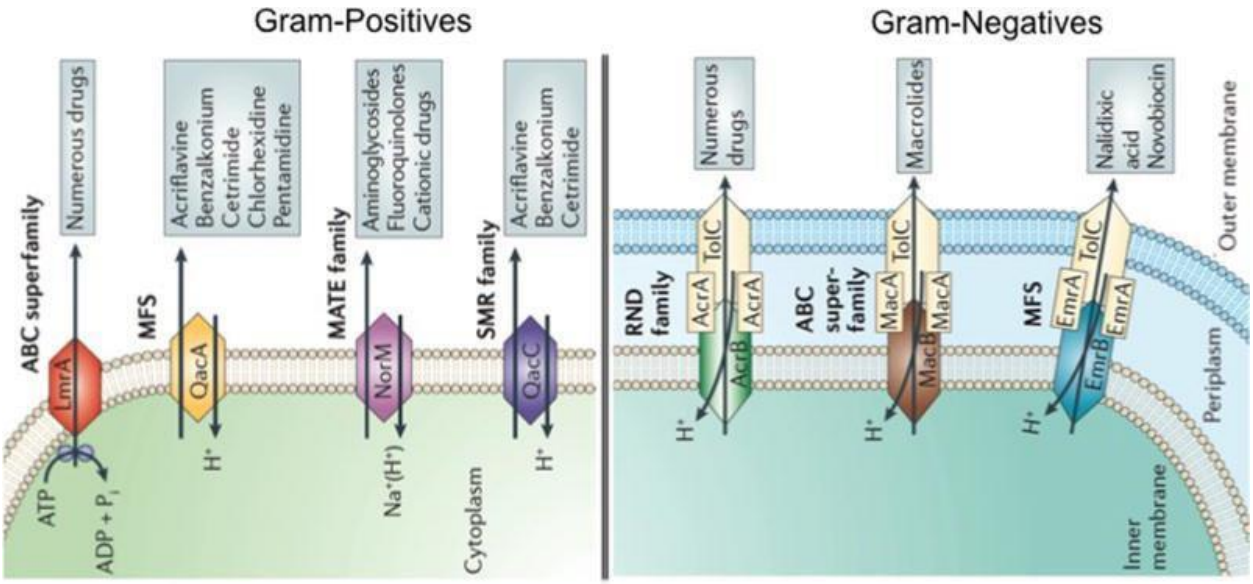

Figure 3. A visual representation of various efflux pumps used in gram positive and gram negative bacteria (Munita & Arias, 2016).

Antibiotic resistance is also a growing problem in *Mycobacterium tuberculosis,* which is acid-fast instead of gram positive or negative (Balaji *et al.,* 2016). Acid-fast positive bacteria, like gram positive and gram negative bacteria, do have peptidoglycan in their cell wall (Alderwick *et al.,* 2015). However, this is not the main component of their cell wall. Acid-fast cell walls are set apart by the large number of mycolic acids they contain, making the cell wall thick, waxy, and difficult for various drugs to permeate (Takayama *et al.,* 2005, Smith *et al.,* 2013). Gram positive bacteria have a thick peptidoglycan cell wall, while gram negative bacteria have only a thin layer of peptidoglycan but also an additional membrane that assists them in drug resistance (Mai-Prochnow *et al.,* 2016). The ability for bacteria to develop methods for antibiotic resistance regardless of cell wall composition shows what a truly widespread problem this is. In comparison to gram negative and gram positive bacteria, there is already a limited range of antibiotics that can treat diseases caused by acid-fast bacteria, such as tuberculosis (Smith *et al.,* 2013). Horizontal gene transfer is also a common way for acid-fast bacteria like *M. tuberculosis*

to become antibiotic resistant, as well as the very slow passage of antibacterial molecules through the very thick cell wall (Smith *et al.,* 2013). These bacteria can also modify drugs to make them less harmful, as well as using various enzymes to break them down. The particularly thick cell wall of acid-fast bacteria already makes it difficult for most antibiotics to permeate (Smith *et al.,* 2013). These many mechanisms of antibiotic resistance make it clear that disease causing acid-fast bacteria are just as much of an increasing problem as gram negative and gram positive bacteria are.

Staphylococcus aureus

Staphylococcus aureus (S. aureus) is a gram positive coccus that can cause significant infections, such as sepsis and infective endocarditis (Teow *et al.,* 2016). These bacteria are currently most well-known for its growing resistance to many types of antibiotics and its ability to cause deadly skin infections (Teow *et al.,* 2016). *S. aureus* related diseases that are difficult to treat with antibiotics are becoming an increasing problem in healthcare settings, specifically methicillin-resistant *Staphylococcus aureus* (MRSA) (Kong *et al.,* 2016). Many strains of *S. aureus* are also exhibiting resistance to the antibiotic vancomycin (McGuinness *et al.,* 2017). Unfortunately, in addition to the strains of *S. aureus* resistant to one specific drug, there are also numerous strains that are multidrug resistant, making them extremely possible to treat and the infections they cause very life-threatening (Onanuga & Temedie, 2011). The lack of response this pathogen has to certain antibiotics is leading healthcare professionals to start to look elsewhere for treatments. MRSA is transmittable from person to person, and based on recent studies, has been found to be evolving at a fast rate (Kong *et al.,* 2016). Due to its growing threat to the world, *S. aureus* is a popular target for research.

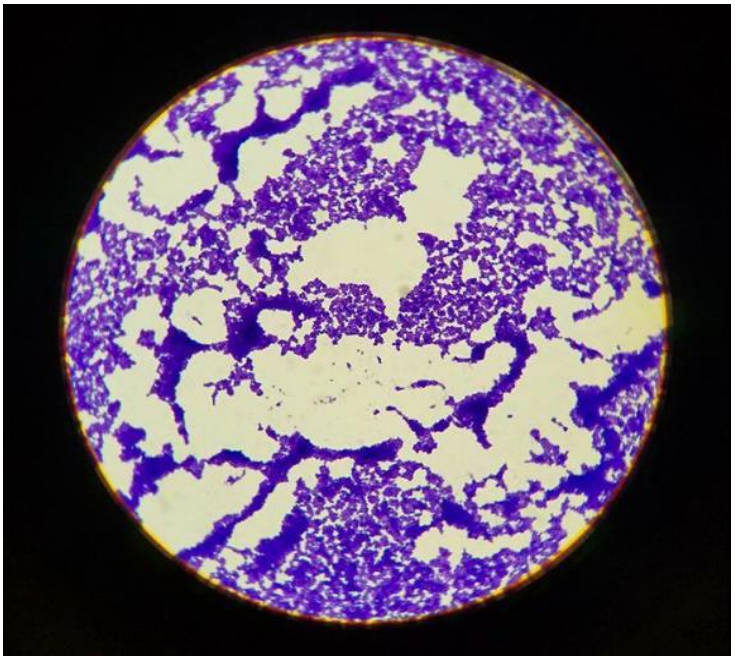

Figure 4. Gram Stain of Gram Positive Coccus *Staphylococcus aureus* (Aponte 2018)

Enterococcus faecalis

Enterococcus faecalis (E. faecalis) is a gram positive coccus that can be found in the gut as part of normal flora of many organisms, including mammals (Van Tyne *et al.*, 2013). Due to shedding through feces, *E. faecalis* can also be found in nature, although it prefers environments that are low in oxygen, such as the intestines (Van Tyne *et al.*, 2013). However, as in the case of other types of bacteria, overuse of antibiotics has resulted in *E. faecalis* becoming a growing health threat. Virulent strains of *E. faecalis* are currently one of the main causes of multidrug resistant infections, such as bacteremia. These infections are made more dangerous by the presence of an exotoxin called cytolysin, which can burst the cells of the host (Van Tyne *et al.*, 2013). Since *E. faecalis* is becoming more resistant to traditional antibiotics, it is also often included in research searching for new antibacterial treatments.

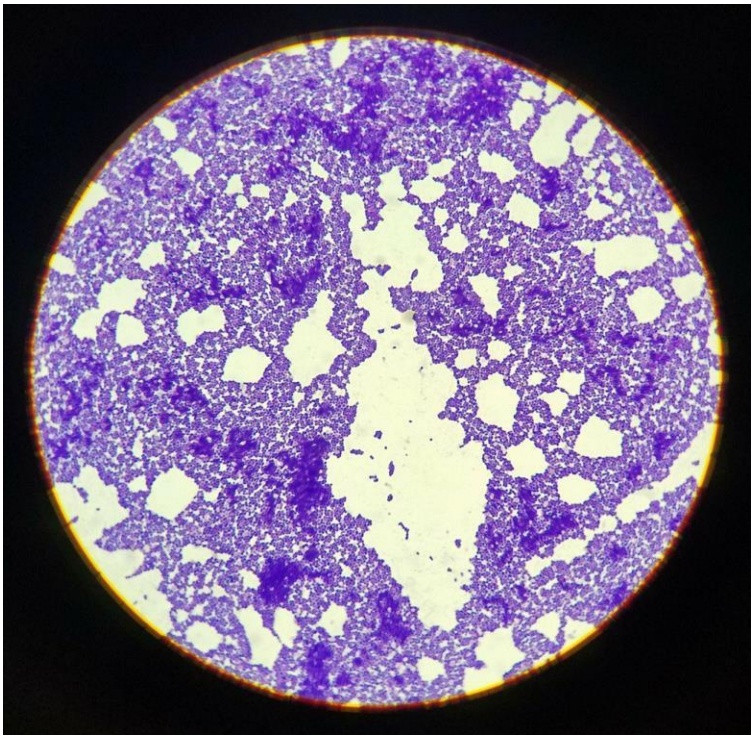

Figure 5. Gram Stain of Gram Positive Coccus *Enterococcus faecalis* (Aponte 2018)

Pseudomonas aeruginosa

Pseudomonas aeruginosa (P. aeruginosa) is a gram negative bacillus often found in soil (Gellatly & Hancock, 2013). It also can cause various infections in humans, and these infections are growing increasingly difficult to treat with current antibiotics. Like other difficult to treat infections, pseudomonal infections are especially a threat in healthcare settings (Gellatly & Hancock, 2013). Some of these infections include bacteremia, ear infections, and, most commonly, respiratory tract infections (Gellatly & Hancock, 2013). The respiratory infections are considered highly dangerous, but unfortunately are difficult to treat. *P. aeruginosa* also has the ability to form biofilms that are difficult to break up. Researchers are looking for treatments that will affect quorum sensing and virulence genes (Gellatly & Hancock, 2013). Drug resistance in *P. aeruginosa* is mainly due to a poorly permeable outer membrane (Gellatly & Hancock,

2013).

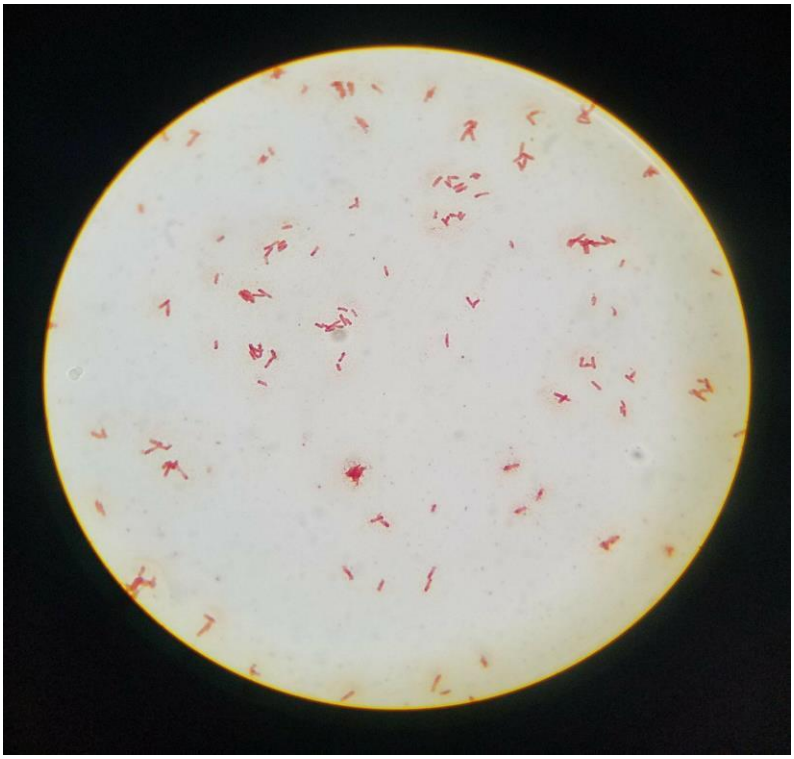

Figure 6. Gram Stain of Gram Negative Bacillus *Pseudomonas aeruginosa* (Aponte 2018)

Escherichia coli

Escherichia coli (E. coli) is a gram negative bacillus that is very commonly found in the healthy gut of humans (Jang *et al.*, 2017). However, certain strains are also associated with gastrointestinal illnesses in humans. The most well-known *E. coli* serovar is O157:H7, which is often in the news for severe food poisoning cases that result in stomach cramping, vomiting, and bloody diarrhea (Jang *et al.*, 2017). While food is a common source of pathogenic *E. coli*, water can contain the bacteria as well. When testing water quality, the presence or absence of *E. coli* is used to determine whether or not the water is contaminated with feces (Jang *et al.*, 2017). Like many other types of bacteria in recent years, *E. coli* has been developing resistance to many of the commonly used antibiotics. This is mainly due to the fact that *E. coli* found within the gut is

subject to the antibiotics consumed by their host (Jang *et al.*, 2017). This poses a threat to human health and is leading researchers to look for new antibacterial treatments.

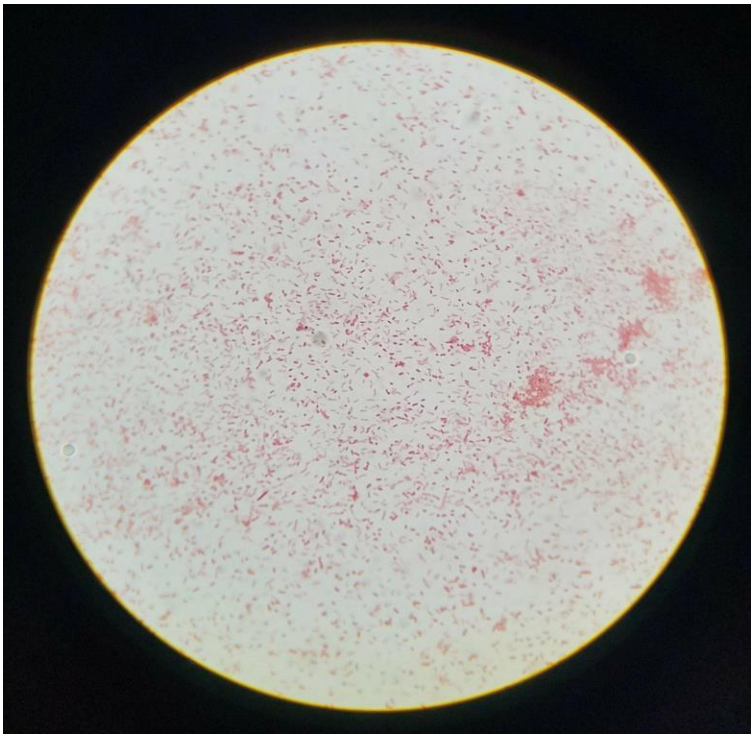

Figure 7. Gram Stain of Gram Negative Bacillus *Escherichia coli* (Aponte 2018)

Mycobacterium smegmatis

Mycobacterium smegmatis (M. smegmatis) is an acid-fast bacteria that does not take up any regular gram staining dyes due to its extremely waxy cell membrane, a result of the presence of mycolic acids (Wu *et al.*, 2018). *M. smegmatis* is the less dangerous relative of *M. tuberculosis*, the causative agent of tuberculosis (Smith, 2003). Researchers can use *M. smegmatis* as a safer way to learn about *M. tuberculosis*. However, it is important to note that they are different from one another. *M. smegmatis* has become a research target because of the growing incidence of antibiotic resistance in cases of tuberculosis (Sotgiu *et al.*, 2015). This is making it necessary for new anti-tubercular drugs to be discovered.

Figure 8: Acid-fast Stain of Acid-fast Positive Bacillus *Mycobacterium smegmatis* (Aponte 2018)

Curcumin

A compound that has provided promising results in terms of being a future antibacterial agent is curcumin, a polyphenolic compound that comes from the spice turmeric (Tyagi *et al.,* 2015) (Figure 9). Turmeric can be consumed and is often used in various foods or mixed into liquids, such as teas. Turmeric is a rhizome, and its scientific name is *Curcuma longa* (Tyagi *et al.,* 2015). Records show that people have been using this rhizome as a broad antimicrobial for quite a long time, and that it does have potential antifungal, antibacterial, and antiviral activity (Moghadamtousi *et al.,* 2014). The earliest known use of turmeric as an herbal medicine goes as far back as about 4000 years ago, where it was used in India. Eventually its use spread throughout Asia, and it was a popular spice in trading (Prasad & Aggarwal, 2011). The antiviral

properties of curcumin have been thoroughly investigated, and it has been determined to work against several viruses. Some of these include human immunodeficiency virus (HIV), where curcumin is believed to inhibit viral replication, and herpes simplex virus 1 (HSV-1), where host cells treated with curcumin are less vulnerable to infection (Kutluay *et al.*, 2008, Flores *et al.*, 2016). Now, scientists look to determine just how effective curcumin can be on different types of drug resistant bacteria.

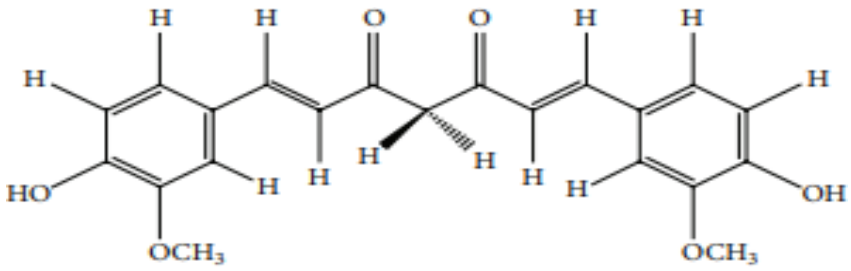

Figure 9. Structure of a Curcumin Molecule (Moghadamtousi *et al.*, 2014)

Currently, methicillin-resistant *Staphylococcus aureus* (MRSA) poses a serious threat to patients who are hospitalized (Gupta *et al.*, 2015). This illness has a very high mortality rate, leaving healthcare professionals and researchers desperate for another solution. *Staphylococcus aureus* that is resistant to antibiotic treatment is thought to be one of the most common causes of infection in the United States (Green *et al.*, 2012). Fortunately, research so far has found curcumin to be especially effective against *Staphylococcus aureus*, which is incredibly important for the future of medicine and the growing issue of antibiotic resistance (Teow *et al.*, 2016). However, much further research is needed before curcumin is ready to be tested as a new treatment. This is also the case for a variety of antibiotic resistant bacteria, including *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Escherichia coli*, which have all been

found to be susceptible to curcumin so far (Tyagi *et al.,* 2015). It has been determined that curcumin's mode of action in both gram positive and gram negative bacteria is to compromise the bacterial membrane, killing the cell (Tyagi *et al.,* 2015). There is also potential for curcumin to be effective against biofilms. Research done with *P. aeruginosa*, another threat to patients staying in a hospital, suggests that curcumin may inhibit genes associated with biofilm formation, such as quorum sensing genes (Moghadamtousi *et al.,* 2014). However, it is also cautioned that it cannot yet be determined definitively that curcumin is effective against *P. aeruginosa.* Overall, however, research done with curcumin as an antibacterial agent shows promising results.

Hispolon

Hispolon, a compound similar in structure to curcumin with only a difference in one aromatic ring, is also being explored by various researchers as a potential antibacterial agent (Ravindran *et al.,* 2010). Hispolon is a polyphenolic compound that has been isolated from various mushrooms, such as *Inonotus hispidus,* and the medicinal mushrooms *Phellinus linteus* and *Phellinus igniarius* (Chethna *et al.,* 2018). It has various derivatives, including hispolon monomethyl ether (HME) (Figure 10) and hispolon pyrazole (HP) (Chethna *et al.,* 2018). It is also considered a curcuminoid derivative (Amalraj *et al.,* 2016). So far, its role as an antibacterial agent is less certain than curcumin. Hispolon is already a known antitumor agent, and it has been suggested to have antiviral properties as well (Chethna *et al.,* 2018). Using this compound as a potential antitumor drug has been the most researched, with much work still needed in terms of hispolon being an antiviral and antibacterial agent. Currently, hispolon has been shown to stop cell proliferation and induce apoptosis in a wide variety of cancers (Kim *et al.,* 2016). The antibacterial effects of hispolon have not been widely explored, unlike the antibacterial effects of

curcumin, especially in terms of how they would work on antibiotic resistant bacteria. However, some early research suggests that various hispolon derivatives may be effective against the *Mycobacterium* genus, leaving it as a possible new treatment for tuberculosis in the future (Balaji *et al.*, 2016). Despite the similarity in structure of hispolon to curcumin, past studies have not found curcumin to be effective against *Mycobacterium* (Balaji *et al.*, 2016). Currently, the hispolon pyrazole derivatives have been found to be especially effective against *Mycobacterium*, likely through preventing synthesis of mycolic acids (Balaji *et al.*, 2019). It is uncertain as to whether or not these specific derivatives have another mechanism to make them active against gram positive and gram negative bacteria. Hispolon derivatives such as hispolon mono methylether are thought to possibly inhibit the production of the enzyme fatty acid synthase II, as well as preventing the synthesis of mycolic acid in *Mycobacterium* (Balaji *et al.*, 2016). This could indicate that these derivatives could be active against gram positive and negative bacteria to some extent, as well as being active against acid-fast bacteria.

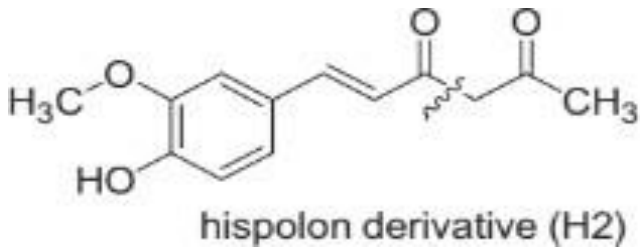

Figure 10. Structure of a Hispolon Derivative Hispolon Mono Methylether (Balaji *et al.*, 2016)

Tuberculosis is a very serious respiratory illness that still affects millions of people worldwide every year. It is caused by the bacterium *Mycobacterium tuberculosis* (Sulis *et al.*, 2014). These bacteria are highly virulent and often cause an inflammatory response in the lungs, resulting in tissue damage. They also have an extremely thick, waxy cell wall, made up of mycolic acid, making treatment difficult (Smith, 2003). Current treatment includes a rigorous antibiotic

regimen, with some of the antibiotics effective against tuberculosis including isoniazid, rifampin, pyrazinamide, kanamycin, and amikacin (Sotgiu *et al.,* 2015). However, despite the wide range of antibiotics available, deaths from tuberculosis are still fairly high, suggesting the need for different antibacterial agents that may work more effectively. This is why it is significant that some research suggests hispolon may be effective against *Mycobacterium* (Balaji *et al.,* 2016). Interestingly enough, even though curcumin is similar in structure, it does not appear to be effective against the *Mycobacterium* genus (Balaji *et al.,* 2016). The true antibacterial effect of hispolon derivatives on other bacteria, such as *S. aureus, E. faecalis, P. aeruginosa,* and *E. coli,* has yet to be determined.

Biofilms

Biofilms, especially in the healthcare setting, have the ability to be very dangerous to humans. Biofilms result from bacteria forming a thick clump on some hard surface and then shielding themselves with extracellular matrix (ECM) (López *et al.,* 2010). The ECM is produced by the bacteria in the biofilm and include polysaccharides, proteins, and some DNA (López *et al.,* 2010). There are many places biofilms can form, and although some may lead to persistent infections in humans, there are some that are not necessarily harmful. An example of a neutral biofilm would be in dental plaques (López *et al.,* 2010). However, there are many cases where the formation of biofilms can lead to infections that are difficult to treat. Many biofilms contain persister cells, which contain toxins thought to block antibiotics from being effective (López *et al.,* 2010). Therefore, biofilms that contain these non-dividing cells are often resistant to a wide range of antimicrobials, making them hard to cure (López *et al.,* 2010).

Biofilm bacteria use a process called quorum sensing in order to communicate with one another (Høiby *et al.,* 2011). Through quorum sensing they are able to turn on genes that allow

for the production of virulence factors, which help make the biofilm more aggressive (Høiby *et al.,* 2011). Mutation is also common in biofilms, increasing the probability of antibiotic resistance. Unfortunately, biofilms are not only resistant to many antibiotics, but they are often able to evade the response by the immune system as well (Høiby *et al.,* 2011). Persister cells are considered one main reason why antibiotic resistance occurs. The ECM itself is also thought to be a source of antibiotic resistance, suggesting that it prevents effective diffusion of drugs into the biofilm (Høiby *et al.,* 2011). Most types of bacteria are able to form biofilms if given the proper environment. *Pseudomonas aeruginosa,* one of the bacteria used in this study, is a common cause of biofilms that threaten human health, particularly in cystic fibrosis patients (Høiby *et al.,* 2011). *P. aeruginosa* often causes lung infections in these patients, and it is capable of causing skin infections as well (López *et al.,* 2010). Naturally, biofilms threaten health are an area of interest in research as scientists try to identify potential treatments that will work more effectively than many of the current antimicrobials.

There is some evidence that antibiotics used in synergy with other antimicrobial compounds, such as curcumin, may be more effective against biofilms than just antibiotics or curcumin alone (Kali *et al.,* 2016). Results from this research so far suggest that when antibiotics and curcumin are used together, curcumin is able to increase antibiotic susceptibility in biofilms, so then the antibiotics are more successful at ending the infection as opposed to when either are working on their own (Kali *et al.,* 2016). Instead of current antibiotics being phased out all together, the solution may be to just use them along with another antibacterial compound. Examples of this have been seen specifically in regard to *Staphylococcus aureus*. Researchers have looked at the use of curcumin and antibiotics together against methicillin resistant *Staphylococcus aureus* (MRSA) strains to see if the curcumin is able to reduce antibiotic resistance (Mun *et al.,* 2013).

S. aureus in particular is an antibiotic resistance threat, making it very popular in research looking for new antimicrobials. Thus far research has found that the use of curcumin with antibiotics such as quinolones and β-lactams against MRSA does in fact make the bacteria more susceptible to the antibiotics by decreasing minimal inhibitory concentration (MIC) (Mun *et al.*, 2013). It is cautioned that more research in this area is needed, particularly with other resistant bacteria besides *S. aureus*, but early results are promising.

Mode of Action

The exact mechanisms by which the antibacterial activity of curcumin and hispolon occur are still to be determined. Since curcumin has been a much larger antibacterial target in current research, scientists do have some ideas as to how this compound stops bacterial growth. Currently results show that curcumin may act by causing leaking in the cell membrane, both in gram negative and gram positive bacteria (Tyagi *et al.*, 2015). This eventually causes the cell to burst and results in death, preventing further reproduction (Tyagi *et al.*, 2015). There is more of a question as to exactly how hispolon works in killing bacteria that are not acid-fast. Studies that have focused on using hispolon compounds to treat tuberculosis suggest that both hispolon and hispolon pyrazole derivatives may target enzymes required to synthesize mycolic acid, which is essential in the *Mycobacterium* cell wall (Balaji *et al.*, 2016, Balaji *et al.*, 2019). If the ability to synthesize mycolic acid is hindered, that will prevent the replication of the bacteria. It is thought that this is done through inhibiting mtFABH, the *Mycobacterium* specific version of the beta-ketoacyl-synthase III enzyme that plays a role in fatty acid synthesis (Balaji *et al.*, 2016, Brown *et al.*, 2005). mtFABH is essential for the synthesis of mycolic acids in acid-fast bacteria, and its inhibition would pose a serious problem for replication (Balaji *et al.*, 2016). Studies suggest that this is one mode of action by hispolon derivatives, and so far the main mode of action by

hispolon pyrazoles (Balaji *et al.*, 2016, Balaji *et al.*, 2019). However, this does not explain the mode of action hispolon compounds may have in gram positive and gram negative bacteria, since their cell walls are made differently. It has also been suggested that the regular hispolon derivatives may block the action of fatty acid synthase II, which could affect fatty acid synthesis in all bacteria (Balaji *et al.*, 2016). This is the mode of action of some current antibiotics (Zhang *et al.*, 2006). Further research focusing on hispolon compounds as antibacterial agents is needed in order to determine its mode of action on these bacteria.

Objectives of This Study:

1) Determine the antibacterial effects of curcumin on *S. aureus, E. faecalis, E. coli, P. aeruginosa,* and *M. smegmatis*.

2) Determine the antibacterial effects of HME on *S. aureus, E. faecalis, E. coli, P. aeruginosa,* and *M. smegmatis*.

3) Determine the antibacterial effects of HP on *S. aureus, E. faecalis, E. coli, P. aeruginosa,* and *M. smegmatis*.

4) Determine if curcumin and HME are effective against biofilms at 50 µM concentrations.

5) Determine if one compound is more effective than the other.

2. Materials and Methods

Preparation of Bacterial Cultures:

Stock cultures of *S. aureus, E. faecalis, E. coli,* and *P. aeruginosa* were prepared by inoculating 15 ml test tubes of previously prepared Brain Heart Infusion (BHI) broth with pure cultures prepared on nutrient agar plates. *M. smegmatis* stock cultures were prepared by inoculating 15 ml test tubes of previously prepared 7H9 neat media with pure cultures prepared

on LB CB+CHX agar plates. All tubes were incubated overnight at 37°C. Stock cultures were then stored at 4°C. The purity of the cultures was checked periodically.

Preparation of Curcumin Stock:

A 10X stock solution of curcumin was prepared by dissolving curcumin powder in dimethylsulfoxide (DMSO). The stock was wrapped in aluminum foil and stored at 4°C. The 10X solution was further diluted as required to produce final concentrations of 25 μM, 50 μM, and 100 μM.

Preparation of HME Stock:

A 10X stock solution of HME was prepared by dissolving HME powder in dimethylsulfoxide (DMSO). The stock was wrapped in aluminum foil and stored at 4°C. The 10X solution was further diluted as required to produce concentrations of 25 μM, 50 μM, and 100 μM.

Preparation of HP Stock:

A 10X stock solution of HP was prepared by dissolving HP powder in dimethylsulfoxide (DMSO). The stock was wrapped in aluminum foil and stored at 4°C. The 10X solution was further diluted as required to produce concentrations of 25 μM, 50 μM, and 100 μM.

DNA Extraction, PCR, and Sequencing to Confirm the Purity of the Cultures

Methods from Lee *et al.,* 2018: In order to determine if the five bacterial cultures were pure and correct, polymerase chain reaction (PCR) was performed using 16S forward and reverse primers, 27F (5'- AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'- ACG GCT ACC TTG TTA CGA CTT-3') respectively. The PCR products were then sequenced and the microorganisms were determined by BLAST search. DNA extraction was first performed on all five microorganisms by adding 500 μl of each bacterium to five separate 1.5 ml microcentrifuge

tubes each containing 100 µl of InstaGene. The tubes were vortexed for 30 seconds and then placed in a 100°C heating block for ten minutes. After the heating period, tubes were immediately placed on ice for one minute and then vortexed for 30 seconds. Tubes were then centrifuged at maximum speed for one minute to separate the extracted DNA into the supernatant. PCR reaction tubes were then prepared for each of the five microorganisms. To each tube, 20 µl of Taq MasterMix, 2 µl of forward primer, 2 µl of reverse primer, 14 µl of nuclease free sterile water, and 2 µl of microbial DNA were added for a total volume of 40 µl (Lee *et al.*, 2018). PCR was completed by the thermalcycler under the following conditions: 95°C for one minute for initial denaturation, 25 cycles of 95°C (10 seconds), 55°C (10 seconds), and 72 °C (10 seconds), and then 72°C for 10 minutes. The PCR products were confirmed through gel electrophoresis and samples were sent out for sequencing. Sequences were copy and pasted into the BLAST computer program to see if the correct bacteria were isolated.

Curcumin Colony Forming Unit (CFU) Assay:

Curcumin treatments were prepared from the 10X stock solution by diluting it to the desired final concentrations of 25 µM, 50 µM, and 100 µM. Untreated cultures were used as a control. Three repeatings were carried out for each condition and each microorganism. For each of the microorganisms, 99 µl of bacteria were treated with 1 µl of each of the three concentrations in microcentrifuge tubes. Treated bacteria were incubated at room temperature for 2 hours (120 minutes). Serial dilutions were prepared using 990 µl of water in 1.5 ml microcentrifuge tubes. For each dilution, 10 µl of treated bacteria were added to the 990 µl of water. Final dilutions for *S. aureus, E. coli,* and *E. faecalis* were 10^{-4}. Final dilutions for *P. aeruginosa* and *M. smegmatis* were 10^{-6}. For each of the 15 samples, 100 µl of each sample was plated onto 15 different nutrient agar plates (3 plates for each microorganism: 25 µM, 50 µM, 100 µM). Control plates

were prepared for each of the five microorganisms, where serial dilutions were performed as mentioned previously and 100 µl of each of the untreated microorganisms were plated on previously prepared nutrient agar plates. Final dilutions for *S. aureus*, *E. coli*, and *E. faecalis*, were 10^{-4}. Final dilutions for *M. smegmatis* were 10^{-6}. Final dilutions for *P. aeruginosa* were 10^{-8}. All plates were inverted and incubated overnight at 37°C. *M. smegmatis* plates were incubated for an additional 6 days. Colony forming units (CFUs) were counted. There were three repeatings for each microorganism. The mean and standard deviation for each microorganism at each treatment concentration were obtained to prepare graphs for CFU data. Each of the five microorganisms were also treated with 2% DMSO to determine the effect of DMSO at this concentration and lower on the bacteria.

HME Colony Forming Unit (CFU) Assay:

HME treatments were prepared from the 10X stock solution by diluting it to the desired final concentrations of 25 µM, 50 µM, and 100 µM. Untreated cultures were used as a control. Three repeatings were carried out for each condition and each microorganism. For each of the microorganisms, 99 µl of bacteria were treated with 1 µl of each of the three concentrations in microcentrifuge tubes. Treated bacteria were incubated at room temperature for 2 hours (120 minutes). The following procedures are the same as described above.

HP Colony Forming Unit (CFU) Assay:

Curcumin treatments were prepared from the 10X stock solution by diluting it to the desired final concentrations of 25 µM, 50 µM, and 100 µM. Untreated cultures were used as a control. Three repeatings were carried out for each condition and each microorganism. For each of the microorganisms, 99 µl of bacteria were treated with 1 µl of each of the three concentrations in

microcentrifuge tubes. Treated bacteria were incubated at room temperature for 2 hours (120 minutes). The following procedures are the same as described above.

% Inhibition Calculations

CFU data collected from all five microorganisms treated with different concentrations of curcumin, HME, and HP was averaged to calculate % inhibition using the formula: **% inhibition= (control-treatment)/control X100**. This was calculated for each microorganism at each treatment concentration for all three compounds. Values were then graphed to compare % inhibition between concentrations and compounds for each of the five microorganisms.

The Effect of Curcumin and HME on the Growth of Microorganisms

Growth curves were performed on *S. aureus, E. faecalis*, *P. aeruginosa*, *E. coli*, and *M. smegmatis* untreated, treated with curcumin (50 µM), and treated with HME (µM). A 48-well plate was used, and three repeatings were carried out for each of the five bacteria under each of the three conditions (untreated, treated with curcumin, and treated with HME). All bacterial samples were diluted with nutrient broth to approximately 0.1 optical density (OD) at 600 nm. The growth was monitored for the five microorganisms untreated, treated with 50 µM curcumin, and treated with 50 µM HME. To each of these wells, 450 µl of bacteria were added. For the untreated, 50 µl of media was added. For the curcumin treated, 50 µl of a 1X curcumin solution at a final concentration of 50 µM was added. For the HME treated, 50 µl of a 1X HME solution at a final concentration of 50 µM HME was added. The three remaining wells were used for negative controls: one with only media (nutrient broth), one with media and curcumin, and one with media and HME. Readings were done every hour, starting at 0 hours and going to 10 hours using the microplate reader. A final reading was taken after 24 hours. The three repeatings for each microorganism under each condition were averaged and graphed on Excel to determine the

overall growth over the 10 hour span. Standard deviation was calculated and shown on the graph as well.

Biofilm Evaluation- Congo Red Assay:

A 24-well Congo Red plate was utilized to test curcumin and HME effectiveness on biofilms. 90 µl of *S. aureus, E. faecalis, E. coli, P. aeruginosa,* and *M. smegmatis* were each incubated with 10 µl of a 1X curcumin solution (100 µM) at room temperature for two hours. In addition, 90 µl of *S. aureus, E. faecalis, E. coli, P. aeruginosa,* and *M. smegmatis* were each incubated with 10 µl of 100 µM HME at room temperature for two hours. Four wells were utilized for each microorganism: a negative control, the microorganism without treatment, the microorganism treated with 50 µM curcumin for two hours, and the microorganism treated with 50 µM HME for two hours. A total of 50 µl of each sample was inoculated into each well. The plate was then incubated at 37°C and checked at 24, 48, and 72 hours.

Biofilm Evaluation- Crystal Violet Assay

For this assay to evaluate action against biofilms, a 48-well plate was prepared. Three repeatings were done for each of the five bacteria (*S. aureus, E. faecalis, E. coli, P. aeruginosa,* and *M.* smegmatis) under each of the three conditions (untreated, treated with curcumin, and treated with HME). All bacterial samples were diluted with nutrient broth to approximately 0.1 optical density (OD). The plate included the five microorganisms untreated, treated with 50 µM curcumin, and treated with 50 µM HME. To each of these wells, 450 µl of bacteria were added. For the untreated samples, 50 µl of media was added. For the curcumin treated, 50 µl of 50 µM curcumin was added. For the HME treated, 50 µl of 50 µM HME was added. The three remaining wells were used for negative controls: one with only media (nutrient broth), one with media and curcumin, and one with media and HME. The plate was incubated at 37°C for four

days to allow formation of biofilms. On the fourth day, liquid was aspirated from all wells and discarded. The wells were washed three times with 250 µl 1X PBS. To each well, 500 µl of 0.1% crystal violet was added and allowed to stain for 30 minutes. The crystal violet was removed and the wells were washed again with 250 µl 1X PBS. The plate was left at room temperature overnight for the stain to dry. Excess stain was wiped up with sterile cotton swabs, and 1 ml of 30% acetic acid was added to each well. The microplate reader was then used to determine optical density at 595 nm for each sample. Averages were taken for each microorganism under each of the three conditions.

Biofilm Evaluation- Resazurin Assay

For this assay to evaluate biofilms, 48-well plate was used, and three repeatings were done for each of the five bacteria under each of the three conditions (untreated, treated with curcumin, and treated with HME). All bacterial samples were diluted with nutrient broth to approximately 0.1 optical density (OD). This growth curve included the five microorganisms untreated, treated with 50 µM curcumin, and treated with 50 µM HME. To each of these wells, 450 µl of bacteria were added. For the untreated, 50 µl of media was added. For the curcumin treated, 50 µl of a 1X curcumin solution at a final concentration of 50 µM was added. For the HME treated, 50 µl of a 1X HME solution at a final concentration of 50 µM was added. The three remaining wells were used for negative controls: one with only media (nutrient broth), one with media and curcumin, and one with media and HME. The plate was incubated at 37°C and 5% CO_2 for four days in order to allow biofilms to grow. After the four days, the liquid was aspirated from each well and discarded. Each well was washed three times with 250 µl 1X PBS. An additional 200 µl 1X PBS was added to each well, along with 200 µl of 20 µM Resazurin. The plate was wrapped in aluminum foil and placed at 4°C overnight. The microplate reader was then used to measure

excitation at 560 nm and emission at 590 nm. Averages were taken for each microorganism under each of the three conditions.

Live-Dead Assay Using Fluorescent Microscopy

Stock plates were prepared for all five microorganisms on nutrient agar plates. To prepare treatment tubes, colonies from the plates were added to 1.5 ml microcentrifuge tubes, resuspended in 5 µl of nutrient broth, and treated with 2 µl of of a 1X curcumin or HME solution at a final concentration of 50 µM for two hours. To prepare the control slides, a loopful of bacteria was taken from the plate for each of the five microorganisms and smeared on the slide. Following this step, 5 µl of 60 Red: 40 Green Live-Dead dye was added directly over the bacterial sample on the slide. For the treatment slides for both curcumin and HME, 5 µl of the treated bacteria were added to the slide, along with 5 µl of Syto®9-Green and Propidium iodide-Red (live-dead dye). All slides were allowed to dry before being focused under the fluorescent microscope and imaged at 40X. This was done for all five microorganisms including a control slide, a curcumin treated slide, and an HME treated slide.

3. Results and Discussion

Bacterial Purity and Species Determination

Following DNA extraction and PCR of each of the five bacteria used in this study, the PCR products were sequences. The sequences obtained were run through the BLAST computer program at the NCBI website in order to determine if the stock samples were pure and correct. Sequencing determined that the proper microorganisms were in fact being used (Figures 11-15).

Figure 11: BLAST Sequencing Results for *S. aureus*

CCCAATAATTCCGGATAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGT
TAGCCGTGGCTTTCTGATTAGGTACCGTCAAAATGTGCACAGTTACTTACACATATGTTCTTCCC
TAATAACAGAGTTTTACGATCCGAAGACCTTCATCACTCACGCGGCGTTGCTCCGTCAGGCTTTC
GCCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAG

TGTGGCCGATCACCCTCTCAGGTCGGCTATGCATCGTTGCCTTGGTAAGCCGTTACCTTACCAAC
TAGCTAATGCAGCGCGGATCCATCTATAAGTGACAGCAAGACCGTCTTTCACTTTTGAACCATGC
GGTTCAAAATATTATCCGGTATTAGCTCCGGTTTCCCGAAGTTATCCCAGTCTTATAGGTAGGTT
ATCCACGTGTTAC GCTTCTCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGG
ATAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATAATATTTTGAA
CCGCATGGTTCAAAAGTGAAAGACGGTCTTGCTGTCACTTATAGATGGATCCGCGCTGCATTAG
CTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATGCATAGCCGACCTGAGAGGGTGATCGGC
CACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAAT
GGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTG

Staphylococcus aureus strain SAPM3 16S ribosomal RNA gene, partial sequence

Staphylococcus aureus strain SAIS4 16S ribosomal RNA gene, partial sequence

Staphylococcus aureus strain SAPM2 16S ribosomal RNA gene, partial sequence

Staphylococcus aureus strain SAIS3 16S ribosomal RNA gene, partial sequence

Figure 11. *Staphylococcus aureus* sequence (via NCBI, blast.ncbi.nlm.nih.gov).

Figure 12: BLAST Sequencing Results for *E. faecalis*

GTTACCGGGGCTGATCTGCGAGTCGTACGCTTCTTTCCTCCCGAGTGCTTGCACTCAATTGGAG
AGAGGAGTGGCGGCCGGGCGAGTATCACGTATGCGGCCTACCCATCAGAGCGGGATTACACTT
GGAAACAGATGCTTATACCGCATAACAGTTTATGCCGCATGCCATATGAGTGAAAGGCGCTTTC
GGGTGTCGCTGATGGATGGCCCCGCGGTGCATTAGCTAGTTGGTGAGATAACGGCTCACCAAGG
CCACCATGCATAGCCGACCTGAAACAGGATTCAACTCTACTGGGACTGACACAGGGTTGAGACT
CCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGC
GTGAGTGAAGAAGACATTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGACGTTACTGA
CCTGAACGTCCCCTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGG
GCAATACGTACGGAGGCAAGCGTTGTGCTGGATTTGATTGGGCGTAAAGCGAGCGCATGGCGT
GTGACTTACGTCTGATGTGACAGCACTCCGGCTTCAACCGGTGCAGGATCATTGTACACTCAGC
ACACTAGAGTGCAACACGAGGCAGAGTGGTATTCACATGATGCTAGCGACTGGAAATGGCACTA
CCTAGTGATGGTAGGGATCACGGGTTGGCTGCACCGACGGCTTCTCTAGGCTCTGGATAACCTC
GGACTGCCTGAAGACCTACGAAAAGGCCGTGGTTCAGCAACTGAGGGATTTCAGTATAACCCTT
GGCTAGGTCAATCGTCAGTAGAACGGAATGGTGGGACTAACGTGGTTCTTGCCATGCGTTCTGC
CGACTTCTATAGCAGTTGGCCTAGCAGTCAAACTGCAACTCCATGCCACGTGCCTGCACTGGCG
GGAGATAACTGTATACTCAGCTACACAGAGATTCGCTCA

Enterococcus faecalis strain KLDS 6.0639 16S ribosomal RNA gene, partial sequence

Enterococcus faecalis strain KLDS 6.0317 16S ribosomal RNA gene, partial sequence

Enterococcus faecalis strain PP02 16S ribosomal RNA gene, partial sequence

Enterococcus faecalis strain PP01 16S ribosomal RNA gene, partial sequence

Enterococcus faecalis strain HBUAS52171 16S ribosomal RNA gene, partial sequence

Enterococcus faecalis strain RCB109 16S ribosomal RNA gene, partial sequence

Figure 12. *Enterococcus faecalis* sequence (via NCBI, blast.ncbi.nlm.nih.gov).

Figure 13: BLAST Sequencing Results for *E. coli*

CAGCACCAGCTACCATGCAGTCGACGGTACAGGAGCAGCTTGCTGCTTCGCTGACGAGTGGCGG
ACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAA
TACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCA
GATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGA
GGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGA
ATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTT
GTAAAGTACTTTCAGCGGGGAGGAAGGGAGTAAAGTTAATACCTTTGCTCATTGACGTTACCCG
CAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAA
TCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGC

TCAACCTGGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGGTAGAATTCC
AGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGCA
CGAAGACTGACGCTCAAGGTGCGAAAGCGTGGCGAGCAAACAGGATTAGATACCCTGGGTAGTT
CCACGCCGTAAAACGATGTCGACTTGGGAGGTTTGTGCCCTTGAGGCGTGCCTTCCGGGAGCTA
ACGCGTTTAAGTCGACCGCCATGGCGGAAGTACGGCTGCAAGGCCTAAAAACTTCTAACTGTAA
TGGAACTGGAGGGGAACTGCACAACCTCTGT

Escherichia coli strain DDH135 16S ribosomal RNA gene, partial sequence

Escherichia coli strain EC1704-2 16S ribosomal RNA gene, partial sequence

Escherichia sp. strain WS35 16S ribosomal RNA gene, partial sequence

Escherichia coli strain CAU2150 16S ribosomal RNA gene, partial sequence

Escherichia sp. CH-22 16S ribosomal RNA gene, partial sequence

Escherichia sp. CH-21 16S ribosomal RNA gene, partial sequence

Escherichia sp. CH-20 16S ribosomal RNA gene, partial sequence

Figure 13. *Escherichia coli* sequence (via NCBI, blast.ncbi.nlm.nih.gov).

Figure 14: BLAST Sequencing Results for *P. aeruginosa*

CGGGGGACAGCTACACATGCAGTCGAGCGGAGAAGGGAGCTTGCTCCTGGATTCAGCGGCGGA
CGGGTGAGTAATGCCTAGGAATCTCGCCTGGTAGTGGGGGATAACGTCCGGAAACGGGCGCTA
ATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCT
AGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAG
AGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGG
AATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGAT
TGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAA
CAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAAT
CGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTCAGCAAGTTGGATGTGAAATCCCCGGGC
TCAAACCTGGGAACTGCATCCCAAAACTACTGAGCTAGAGTACGGGTAGAGGGTGGTGGAATTT
CCCTGTGTAGCCGGTGAAATGCGTAGAATAATAGGTAGGAACATCCAGTGTCGGAAGCGACCAC
CTTGGACTGATACCTGACACTGAAGTGGGCGAAAGCGTGGGGAGCAACAGAATAGATACCTTGC
TAGTCCACGCCGTAACGATGTCCGACTAGTCGTCGGAATCCTGGAACTTTATGCGCAGCTTAAC
GCATAGTGCACCGCTGGTATACGTCGCAGGGGTTTAC

Pseudomonas aeruginosa strain Ki2y 16S ribosomal RNA gene, partial sequence

Pseudomonas aeruginosa strain MS-5 16S ribosomal RNA gene, partial sequence

Pseudomonas aeruginosa strain A1816 16S ribosomal RNA gene, partial sequence

Pseudomonas aeruginosa strain QK-4 16S ribosomal RNA gene, partial sequence

Pseudomonas aeruginosa strain BR1-B 16S ribosomal RNA gene, partial sequence

Pseudomonas aeruginosa strain SMGV-11 16S ribosomal RNA gene, partial sequence

Figure 14. *P. aeruginosa* sequence (via NCBI, blast.ncbi.nlm.nih.gov).

Figure 15: BLAST Sequencing Results for *M. smegmatis*

CTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGC
AAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAC
AGACGAAGCGCAAGTGACGGTATGTGCAGAAGAAGGACCGGCCAACTACGTGCCAGCAGCCGC
GGTAATACGTAGGGTCCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTG
TCGCGTTGTTCGTGAAAACTCACAGCTTAACTGTGGGCGTGCGGGCGATACGGGCAGACTAGAG
TACTGCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACAC
CGGTGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA
CAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGTACTAGGTGTGGGTTTCCTTCCT
TGGGATCCGTGCCGTAGCTAACGCATTAAGTACCCCGCCTGGGGAGTACGGCCGCAAGGCTAAA

ACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGATTAATTCGATGCAACG
CGAAGAACCTTACCTGGGTTTGACATGCACAGGACGCCGGCAGAGATGTCGGTTCCCTTGTGGC
CTGTGTGCAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
ACGAGCGCAACCCTTGTCTCATGTTGCCAGCACGTTATGGTGGGGACTCGTGAGAGACTGCCGG
GGTCAACTCGGAGGAAGGTGGGGATGACGTCAAGTCATC

Mycobacterium smegmatis strain PA.2 16S ribosomal RNA gene, partial sequence

Mycobacterium smegmatis strain C4.1 16S ribosomal RNA gene, partial sequence

Mycobacterium smegmatis strain C5.1b 16S ribosomal RNA gene, partial sequence

Mycobacterium smegmatis strain C5.1a 16S ribosomal RNA gene, partial sequence

Mycobacterium smegmatis strain C2.3 16S ribosomal RNA gene, partial sequence

Figure 15. *M. smegmatis* sequence (via NCBI, blast.ncbi.nlm.nih.gov).

Screening Effectiveness of Curcumin on Bacterial Cells by CFU Assays

To determine whether or not curcumin would be at all effective against the various types of bacteria used in this study, each of the five microorganisms were initially treated with varying concentrations of curcumin (25 μM, 50 μM, and 100 μM) for two hours (120 minutes) at room temperature. The plates were incubated overnight, and colonies were counted on each plate, including all control and treated plates. This was repeated for each microorganism three times. The results indicated that limited colonies were seen on treated *S. aureus, E. faecalis, P. aeruginosa,* and *E. coli* for all concentrations compared to all control plates. *M. smegmatis* did not seem to be inhibited by curcumin (Figure 16). These results suggest that curcumin is effective against four of the five studied microorganisms, and that acid-fast bacteria may be resistant to curcumin treatment.

Figure 16: Treatment of *Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa,* and *Mycobacterium smegmatis* with Varying Concentrations of Curcumin

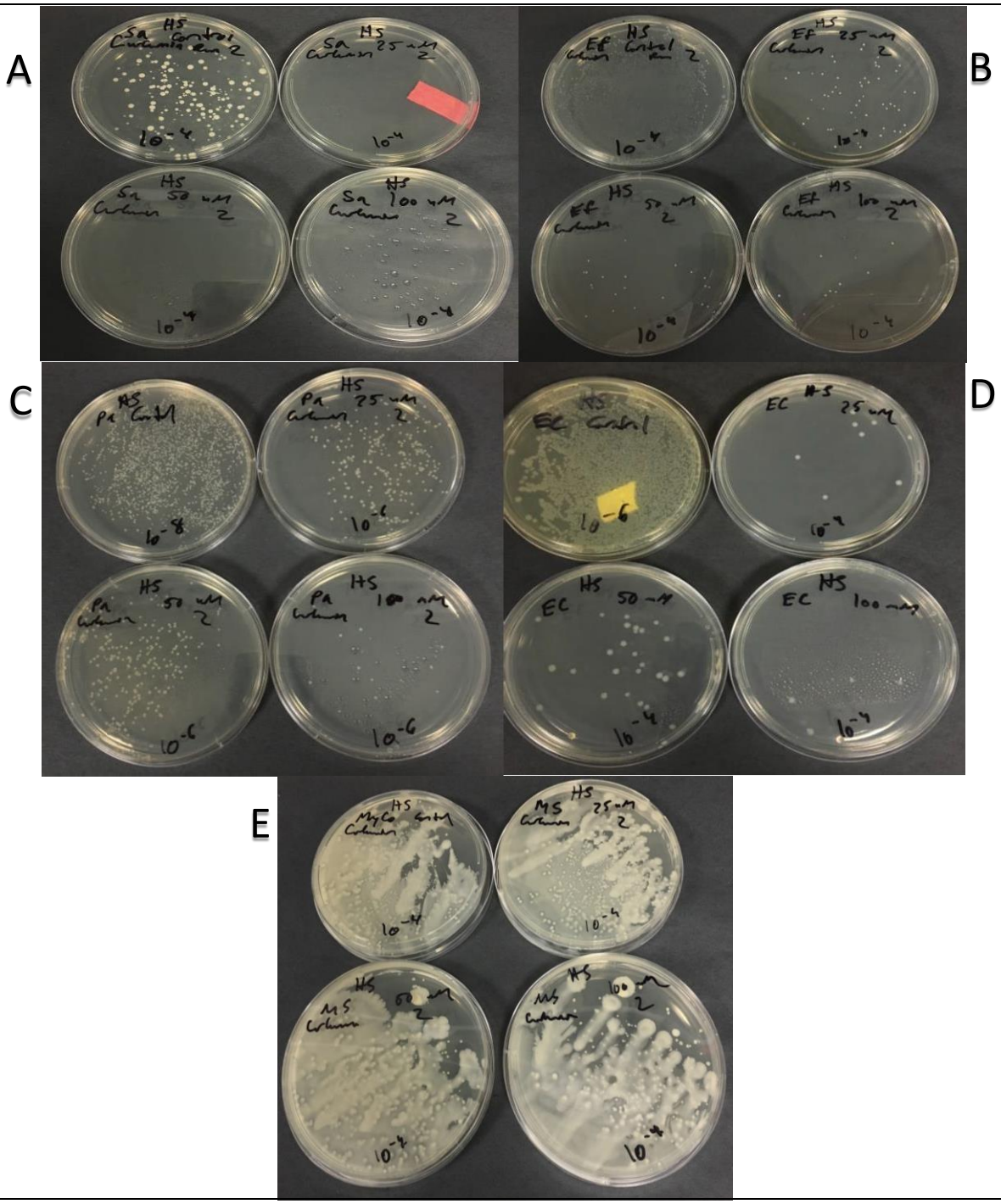

Figure 16. Treatment with curcumin for two hours at room temperature was done on *Staphylococcus aureus* (A), *Enterococcus faecalis* (B), *Pseudomonas aeruginosa* (C), *Escherichia coli* (D), and *Mycobacterium smegmatis* (E) to determine the effectiveness of the compound as an antibacterial agent.

Screening Effectiveness of HME on Bacterial Cells by CFU Assays

In order to determine whether or not hispolon would also be effective against the various types

of bacteria used in this study, each of the five microorganisms were initially treated with varying concentrations of HME (25 μM, 50 μM, and 100 μM) for two hours (120 minutes) at room temperature. The plates were incubated overnight and then compared to control plates to determine whether or not HME inhibited bacterial growth. A colony forming unit (CFU) assay was performed and colonies were counted on each plate, including all control and treated plates. This was repeated for each bacteria 3 times. Limited growth was seen on all treated bacteria at all concentrations: *S. aureus, E. faecalis, P. aeruginosa, E. coli*. and *M. smegmatis* (Figure 17). These results suggest that HME is potentially effective in preventing the growth of all five microorganisms used in this study.

Figure 17: Treatment of *Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa, Escherichia coli,* and *Mycobacterium smegmatis* with Varying Concentrations of HME

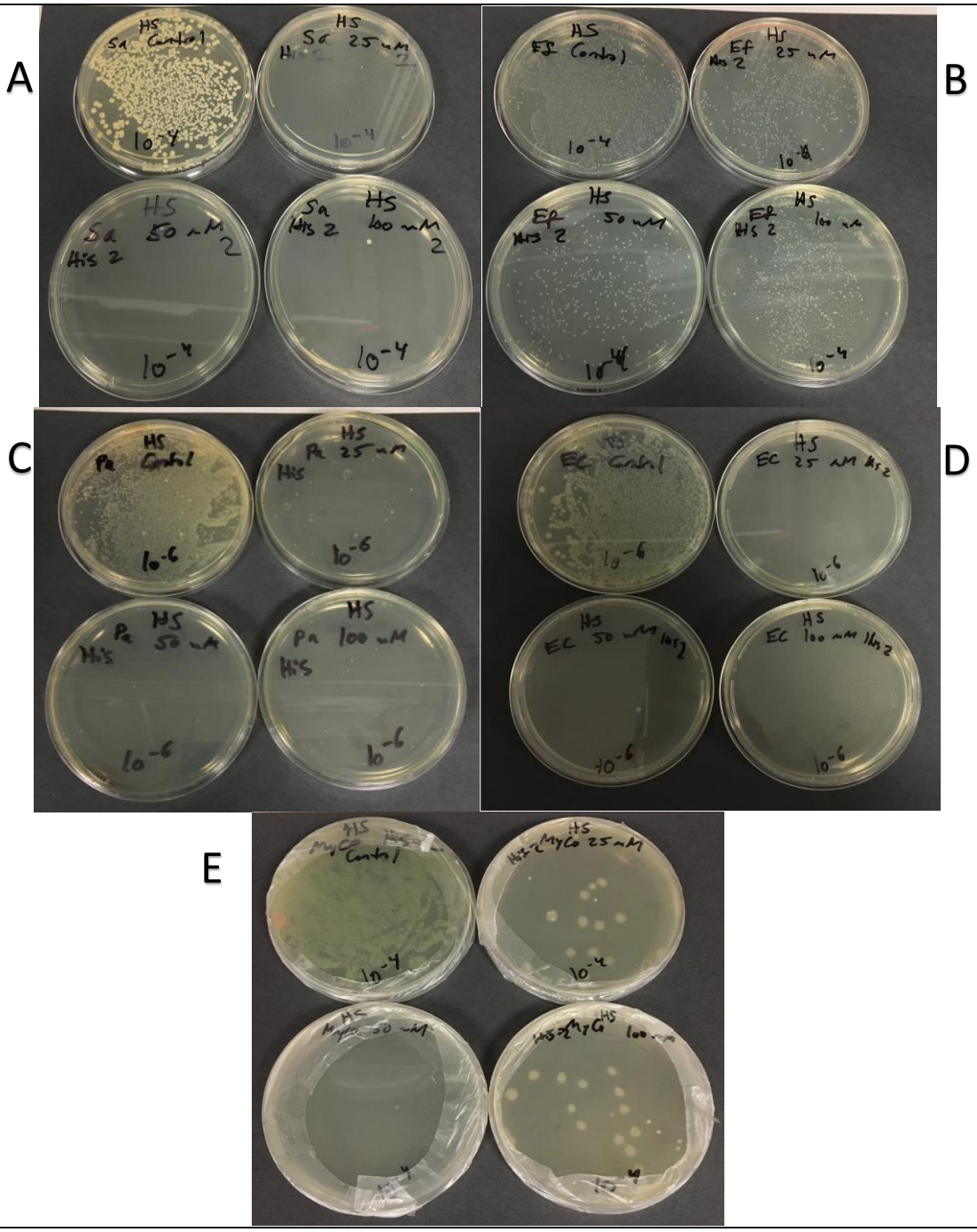

Figure 17. Treatment with hispolon-2 for two hours at room temperature was done on *Staphylococcus aureus* (A), *Enterococcus faecalis* (B), *Pseudomonas aeruginosa* (C), *Escherichia coli* (D), and *Mycobacterium smegmatis* (E) to determine the effectiveness of the compound as an antibacterial agent.

Screening Effectiveness of HP on Bacterial Cells by CFU Assays

To determine if another hispolon derivative, hispolon pyrazole (HP), was effective against the various types of bacteria used in this study, each of the five microorganisms were initially treated with varying concentrations of HP (25 μM, 50 μM, and 100 μM) for two hours (120 minutes) at room temperature. The plates were incubated overnight and then compared to control plates to determine whether or not HP inhibited bacterial growth. A colony forming unit (CFU) assay was performed and colonies were counted on each plate, including all control and treated plates. This was repeated for each bacteria 3 times. This compound did not appear to significantly inhibit *S. aureus*, *E. faecalis*, *E. coli*, and *P. aeruginosa*, since treated plates had almost as many, if not more, colonies than control plates. Only *M. smegmatis* was significantly inhibited by this compound (Figure 18). These results suggest that HP is not as effective as HME in preventing bacterial growth, with the exception of *M. smegmatis*.

Figure 18. Treatment of *Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa, Escherichia coli,* and *Mycobacterium smegmatis* with Varying Concentrations of HP

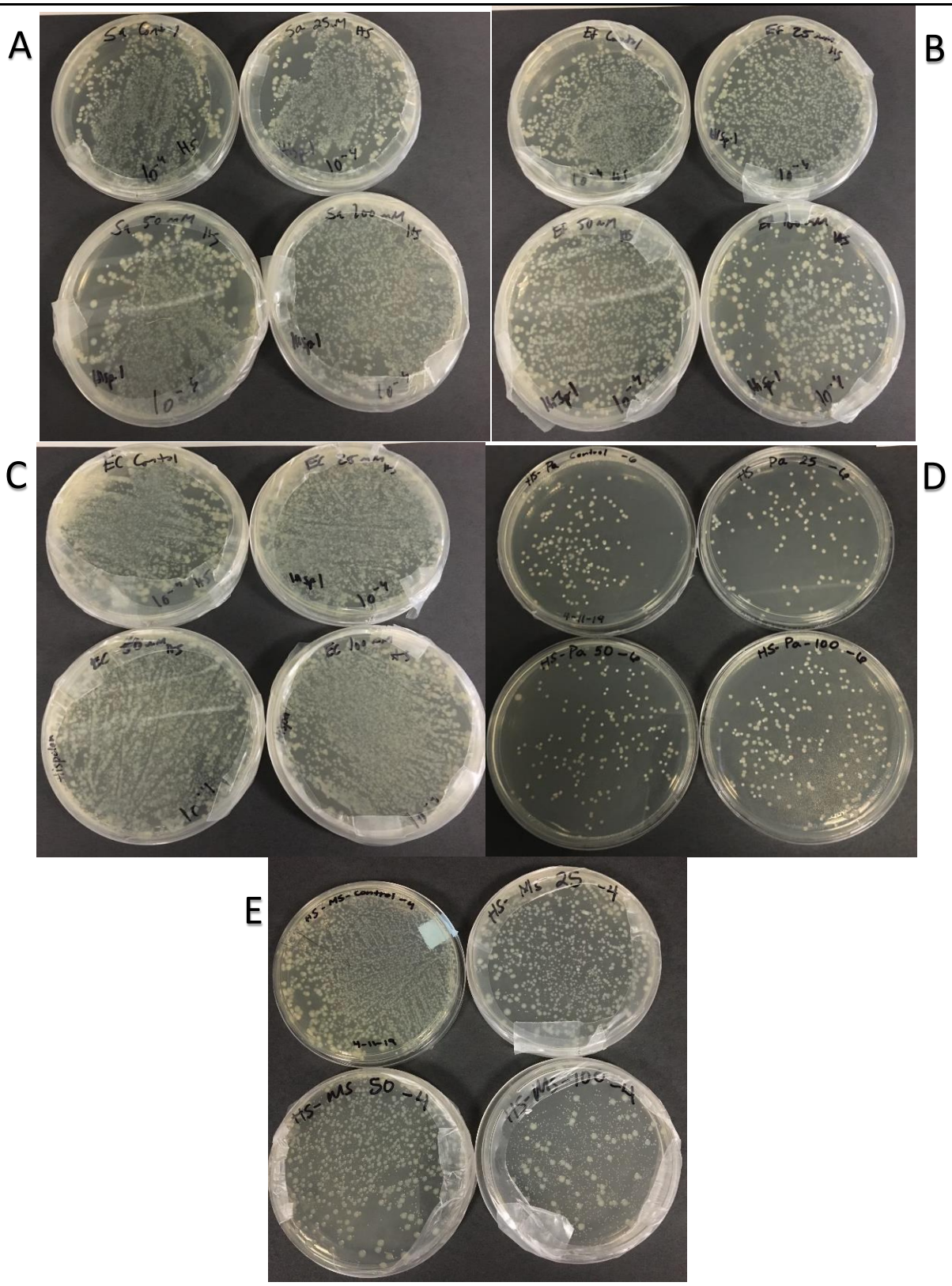

Figure 18. Treatment with HP for two hours at room temperature was done on *Staphylococcus aureus* (A), *Enterococcus faecalis* (B), *Pseudomonas aeruginosa* (C), *Escherichia coli* (D), and *Mycobacterium smegmatis* (E) to determine the effectiveness of the compound as an antibacterial agent.

Colony Forming Unit (CFU) Assay Graphs for Curcumin, HME, and HP

All CFU data collected from control plates as well as curcumin, HME, and HP treated plates were averaged and converted into graphs comparing colony forming units among control plates, 25 μM treated plates, 50 μM treated plates, and 100 μM treated plates for each microorganism. CFU data for each microorganism treated with each compound was graphed separately. When comparing CFU data for bacteria treated with curcumin (Figure 19) and bacteria treated with HME (Figure 20), it was determined that bacteria treated with curcumin tended to have fewer CFUs than bacteria treated with HME. Both compounds worked extremely well against *S. aureus* in particular (Figure 19A, Figure 20A), with average growth of 45 CFUs/10^4 for control plates and average CFUs of less than one for curcumin treated plates. HME treated plates all had average CFUs two or lower. Both treatments also worked exceptionally well on *P. aeruginosa*. For all bacteria treated with both compounds besides *M. smegmatis* treated with curcumin, treated plates at all three concentrations had fewer CFUs than control plates. However, HP was considerably less successful against *S. aureus, E. faecalis, E. coli,* and *P. aeruginosa,* with treated plates having almost as many, and in some cases more, CFUs than the control plates (Figure 21). However, HP did seem to be effective against *M. smegmatis*, which had hundreds of colonies on the control plates and averages below two hundred on all of the treated plates.

Figure 19: CFUs for *Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa, Escherichia coli,* and *Mycobacterium smegmatis* Treated with Curcumin

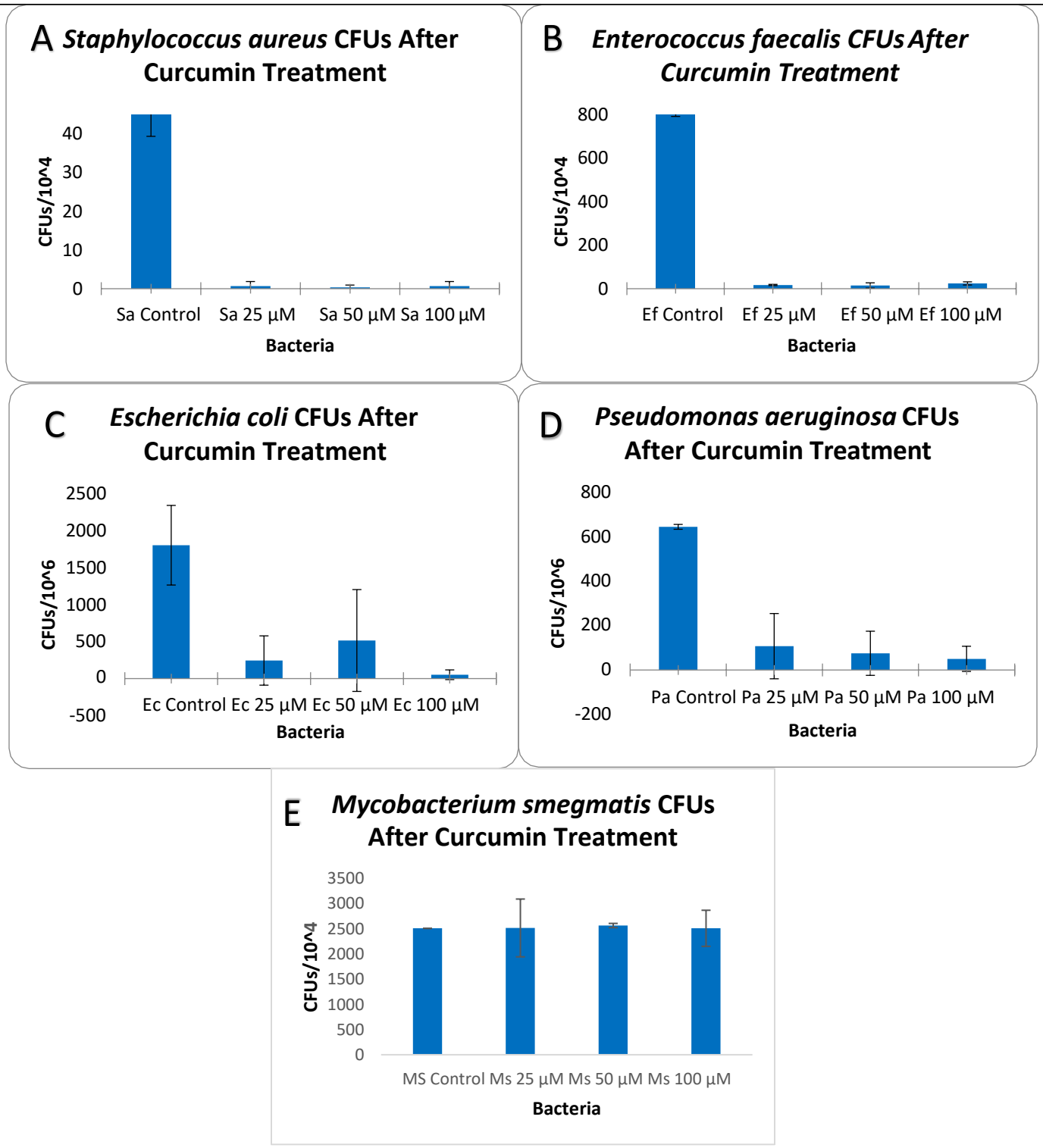

Figure 19. Colony Forming Units (CFUs) for *Staphylococcus aureus* (A), *Enterococcus faecalis* (B), *Escherichia coli* (C), *Pseudomonas aeruginosa* (D), and *Mycobacterium smegmatis* (E) treated for two hours with varying concentrations of curcumin.

Figure 20: CFUs for *Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa, Escherichia coli,* and *Mycobacterium smegmatis* Treated with HME

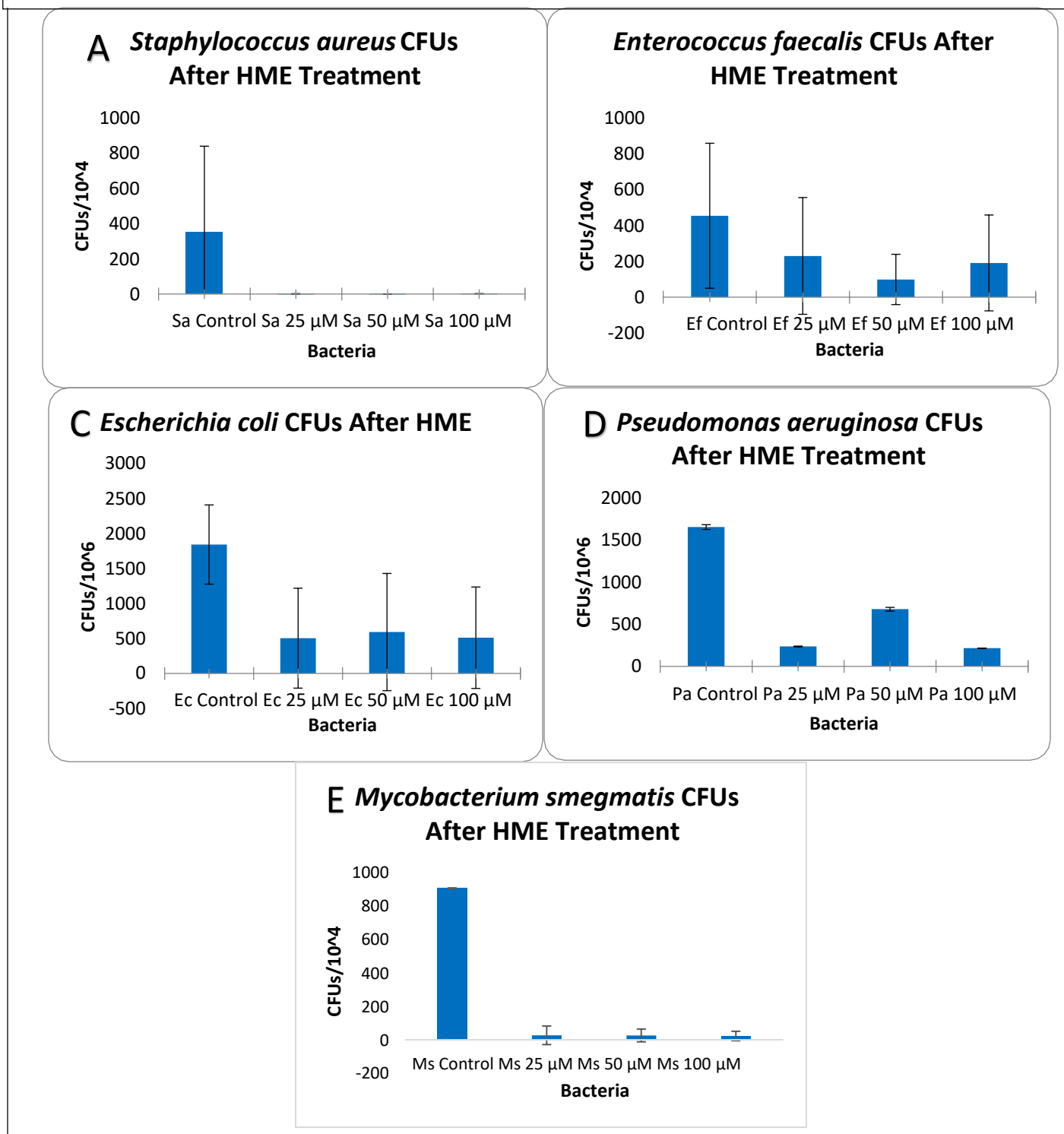

Figure 20. Colony Forming Units (CFUs) for *Staphylococcus aureus* (A), *Enterococcus faecalis* (B), *Escherichia coli* (C), *Pseudomonas aeruginosa* (D), and *Mycobacterium smegmatis* (E) treated for two hours with varying concentrations of HME.

Figure 21: CFUs for *Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa, Escherichia coli,* and *Mycobacterium smegmatis* Treated with HP

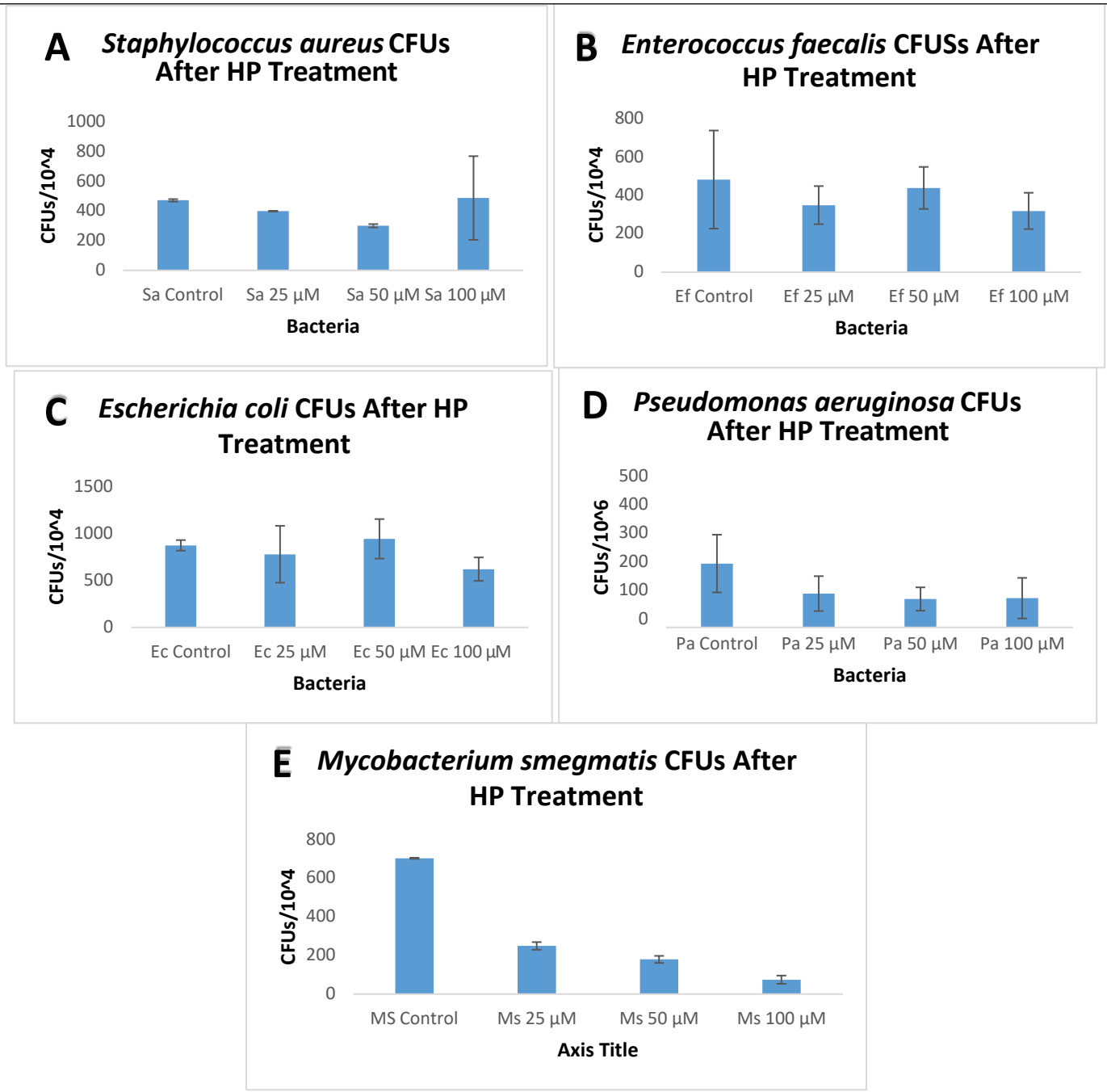

Figure 21. Colony Forming Units (CFUs) for *Staphylococcus aureus* (A), *Enterococcus faecalis* (B), *Escherichia coli* (C), *Pseudomonas aeruginosa* (D), and *Mycobacterium smegmatis* (E) treated for two hours with varying concentrations of HP.

% Inhibition of Curcumin, HME, and HP

CFU data collected for curcumin, HME, and HP treated bacteria was used to calculate %

inhibition. Each run for each of the five bacteria was averaged to make these calculations. % inhibition was calculated using the formula **% Inhibition= (Control-Treatment)/Control X 100.** This was calculated for each of the five bacteria at each treatment concentration (25 μM, 50 μM, and 100 μM) for all compounds. Graphs were made all bacteria for all compounds. When comparing % inhibition data for bacteria treated with curcumin (Figure 22, Table 1) with bacteria treated with HME (Figure 23, Table 2), curcumin was found to have an overall higher % inhibition than HME. The % inhibition for *S. aureus* treated with curcumin was almost 99% for all three concentrations (Figure 22A), and % inhibition values for *E. faecalis* treated with curcumin (Figure 22B) were between 96 and 98%. The % inhibition values for *E. coli* treated with curcumin ranged between 71% and 97%, while the % inhibition values for *P. aeruginosa* treated with curcumin ranged from 83% to 92%. While HME was at least somewhat effective on each of the microorganisms, the % inhibition values overall were lower than they were for curcumin treated bacteria. The % inhibition values for *S. aureus* were all at 99% (Figure 23A), but the % inhibition values of *E. faecalis, E. coli,* and *P. aeruginosa* were less impressive (23B, 23C, 23D). It appeared to be least effective on *E. faecalis,* whose % inhibition values only ranged from about 49% to 60%. For HP, % inhibition values overall were very low, with almost all values for all bacteria besides *M. smegmatis* falling under 50% (Figure 24, Table 3). HME produced significantly higher % inhibition values than HP.

Overall, % inhibition values for all three compounds showed that while both curcumin and HME are highly effective at preventing colony forming units, HP is not. Curcumin worked on all bacteria but *M. smegmatis*, HME worked on all five bacteria, and HP only worked on *M. smegmatis*. The ability of hispolon derivatives to work so well on *M. smegmatis* so well sets it apart from curcumin, which appears to work well against gram positive and gram negative

bacteria but not against acid-fast bacteria. Values for curcumin were highest for gram positive bacteria, while HME did not have a pattern based on cell wall composition.

Figure 22: % Inhibition for *Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa, Escherichia coli,* and *Mycobacterium smegmatis* Treated with Curcumin

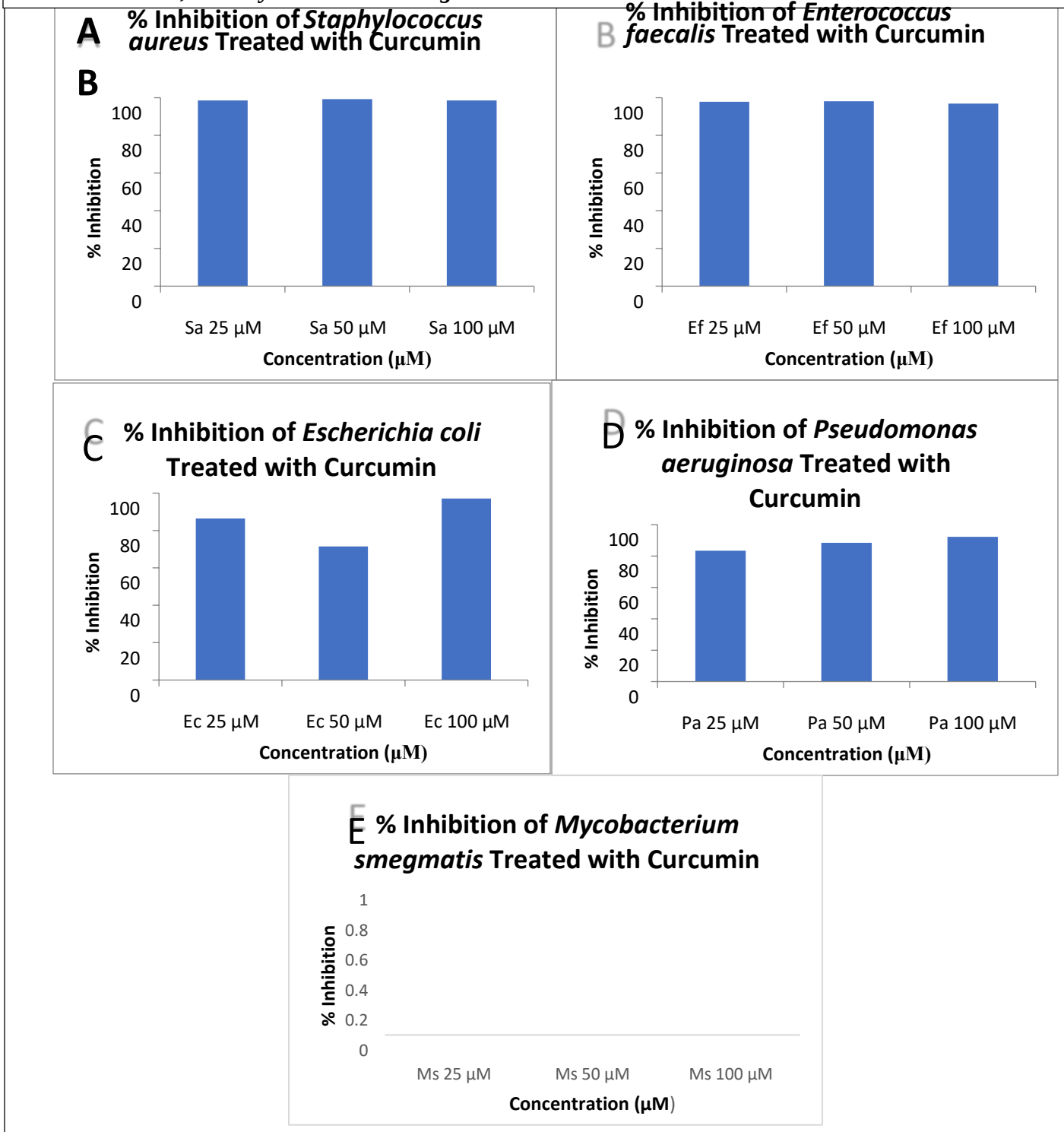

Figure 22. % inhibition calculations for *Staphylococcus aureus* (A), *Enterococcus faecalis* (B), *Escherichia coli* (C), *Pseudomonas aeruginosa* (D), and *Mycobacterium smegmatis* (E) treated for two hours with varying concentrations of curcumin.

Table 1. % Inhibition Calculations for Curcumin Treated Bacteria

Bacteria	% Inhibition
Sa 25 µM	98.52
Sa 50 µM	99.27
Sa 100 µM	98.52
Ef 25 µM	97.91
Ef 50 µM	98.16
Ef 100 µM	96.95
Ec 25 µM	86.42
Ec 50 µM	71.44
Ec 100 µM	97.11
Pa 25 µM	83.41
Pa 50 µM	88.43
Pa 100 µM	92.28
Ms 25 µM	0
Ms 50 µM	0
Ms 100 µM	0

Table 1 shows the % inhibition calculations for bacteria treated with curcumin at each concentration based off of average CFU count.

Figure 23: % Inhibition for *Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa, Escherichia coli,* and *Mycobacterium smegmatis* Treated with HME

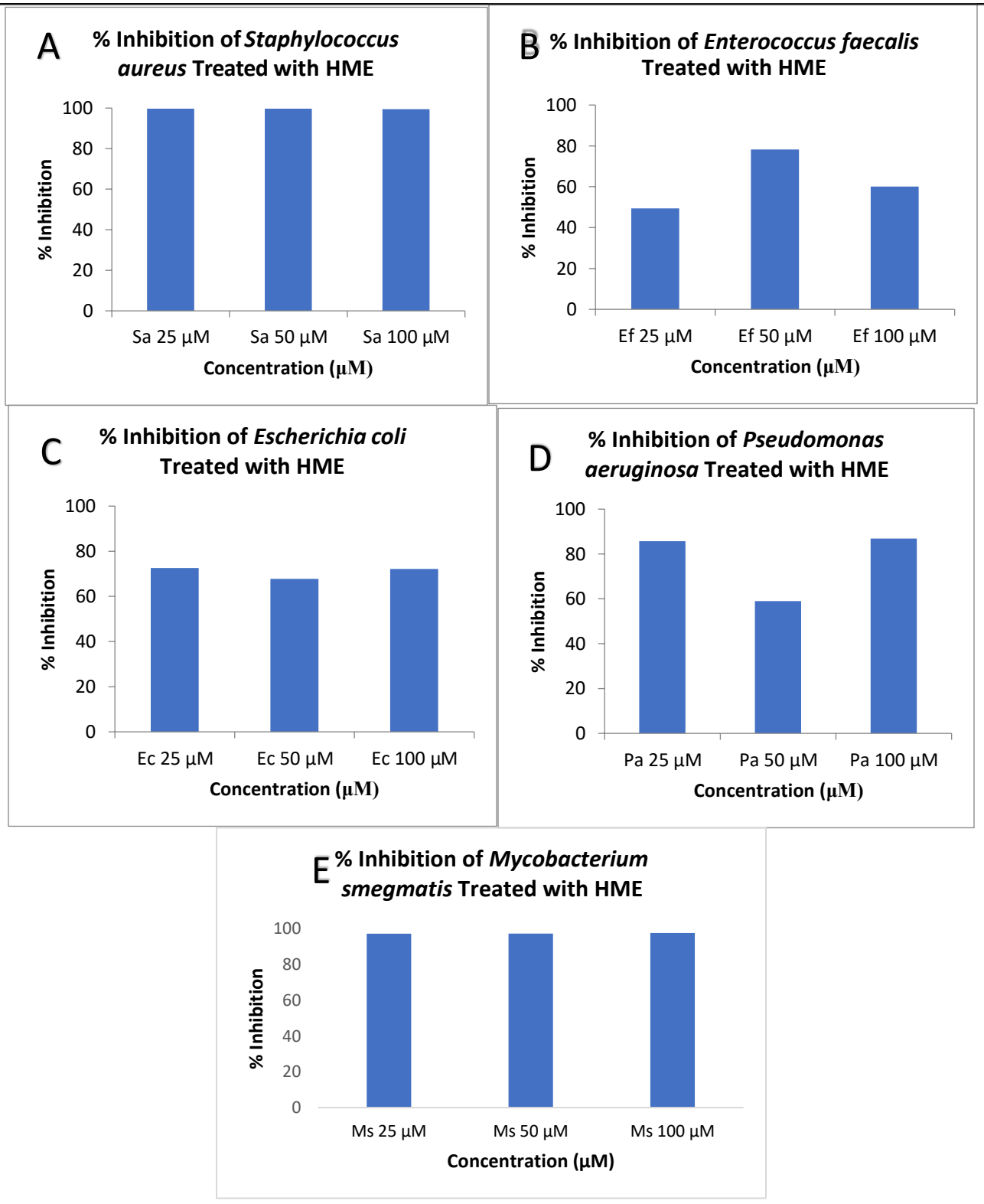

Figure 23. % inhibition calculations for *Staphylococcus aureus* (A), *Enterococcus faecalis* (B), *Escherichia coli* (C), *Pseudomonas aeruginosa* (D), and *Mycobacterium smegmatis* (E) treated for two hours with varying concentrations of HME.

Table 2. % Inhibition Calculations for HME Treated Bacteria

Bacteria	% Inhibition
Sa 25 µM	99.71
Sa 50 µM	99.71
Sa 100 µM	99.43
Ef 25 µM	49.34
Ef 50 µM	78.19
Ef 100 µM	60
Ec 25 µM	72.55
Ec 50 µM	67.77
Ec 100 µM	72.17
Pa 25 µM	85.67
Pa 50 µM	59
Pa 100 µM	86.87
Ms 25 µM	97
Ms 50 µM	97.1
Ms 100 µM	97.4

Table 2 shows the % inhibition calculations for bacteria at each concentration based off of average CFU count.

Figure 24: % Inhibition for *Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa, Escherichia coli,* and *Mycobacterium smegmatis* Treated with HP

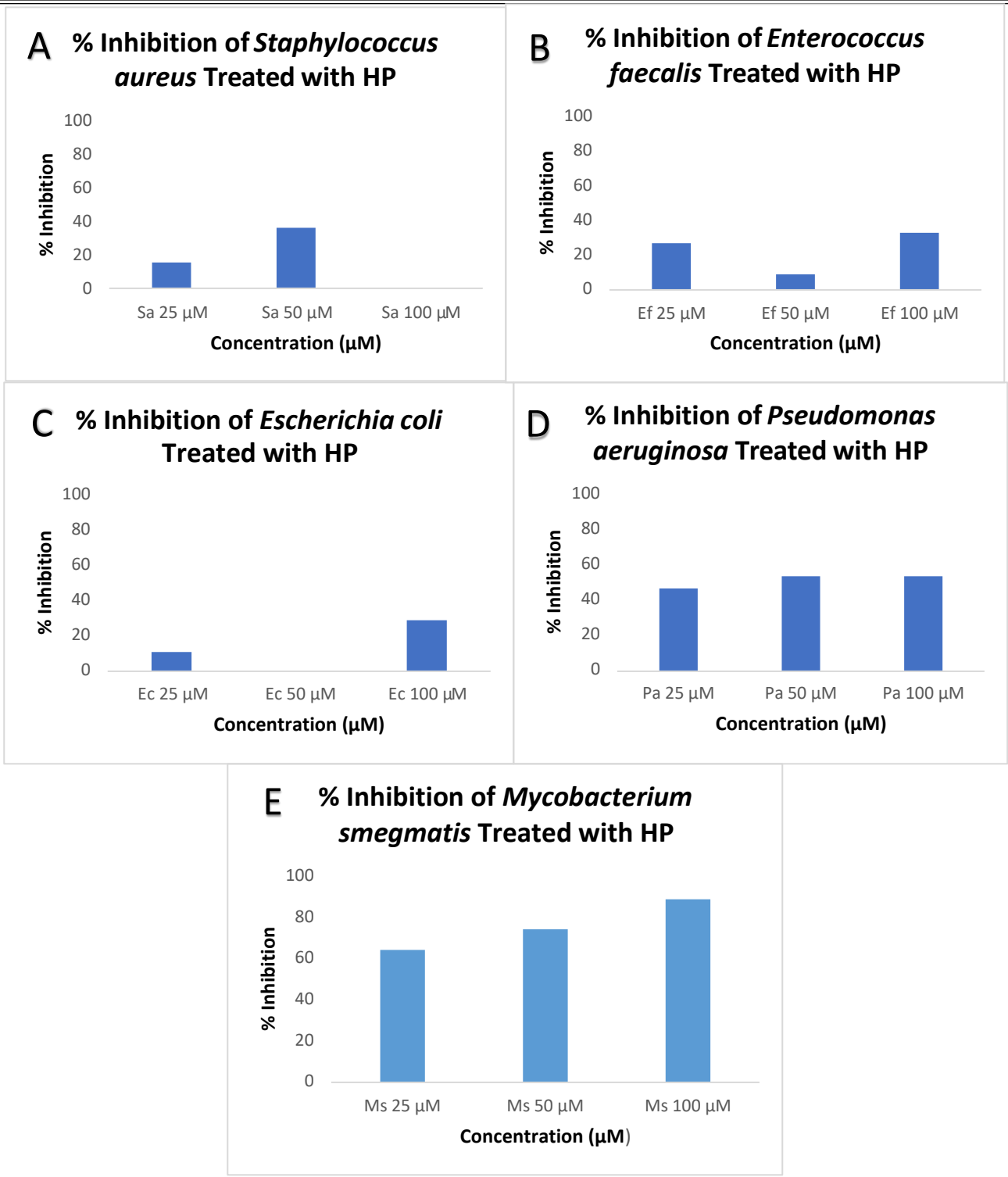

Figure 24. % inhibition calculations for *Staphylococcus aureus* (A), *Enterococcus faecalis* (B), *Escherichia coli* (C), *Pseudomonas aeruginosa* (D), and *Mycobacterium smegmatis* (E) treated for two hours with varying concentrations of HP.

Table 3. % Inhibition Calculations for HP Treated Bacteria

Bacteria	% Inhibition
Sa 25 µM	15.4
Sa 50 µM	36
Sa 100 µM	0
Ef 25 µM	27
Ef 50 µM	9
Ef 100 µM	33
Ec 25 µM	11
Ec 50 µM	0
Ec 100 µM	29
Pa 25 µM	47
Pa 50 µM	54
Pa 100 µM	54
Ms 25 µM	64.4
Ms 50 µM	74.4
Ms 100 µM	89

Table 3 shows the % inhibition calculations for bacteria at each concentration based off of average CFU count.

Evaluation of the Effect of Curcumin and HME on the Growth of Bacteria

Growth was monitored for *S. aureus, E. faecalis, P. aeruginosa, E. coli*, and *M. smegmatis* to see if curcumin and HME had effects on bacterial growth. This examined growth of each microorganism with no treatment in order to compare the growth of the untreated microorganisms to the growth of the same microorganisms treated with curcumin and HME. A 48 well plate was used to include all samples. For this experiment, readings were taken every hour for ten hours. One last reading was taken after twenty-four hours. In the same plate as the untreated bacteria, the same microorganisms were treated with curcumin and with HME. The treated bacteria were expected to have slower growth than the untreated bacteria, if any steady growth at all. This was measured in optical density (OD) at 600 nm. Three runs were carried out for each microorganism in this study, for both treated and untreated bacteria. The average OD

values throughout the ten hours were graphed for each microorganism under each of the three conditions. It must be noted that although all of the microorganisms were diluted to an initial 0.1 OD before being plated, curcumin is very heavily pigmented and affects OD readings, despite normalization, making the starting OD of curcumin bacteria higher than untreated or HME treated bacteria.

a. Staphylococcus aureus

Staphylococcus aureus exhibited normal growth when untreated, shown by Figure 25. OD increased steadily throughout the ten hour period, starting at an OD reading around 0.2 and finishing at around 0.6. For the curcumin treated *S. aureus*, OD remained nearly stagnant for the ten hour period after dropping slightly initially, and HME treated *S. aureus* showed no growth and a steadier decrease in OD over the ten hours. These results suggest that both curcumin and HME have antibacterial effects against *S. aureus* growth.

Figure 25: Growth Curve for Treated and Untreated *Staphylococcus aureus*

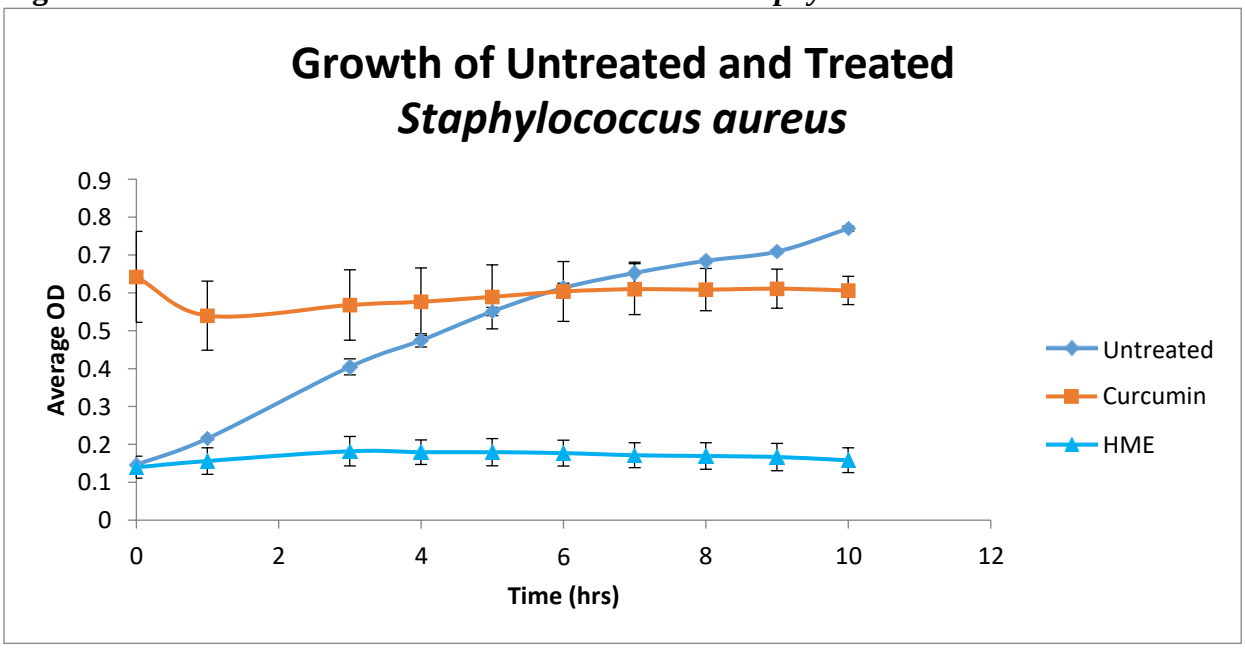

Figure 25. Average OD with standard deviation for untreated, curcumin treated, and HME treated *Staphylococcus aureus*.

b. *Enterococcus faecalis*

Enterococcus faecalis is a slower growing microorganism, and growth for the untreated bacteria over the ten hour reading period was less than for the other microorganisms used in this study (Figure 26). A very long lag phase of around 6 hours was observed. OD for *E. faecalis* treated with curcumin dropped initially and then remained stagnant. *E. faecalis* treated with HME did not see much of a change in OD over time. The results indicated that the growth was inhibited by both curcumin and HME.

Figure 26: Growth Curve of Treated and Untreated *Enterococcus faecalis*

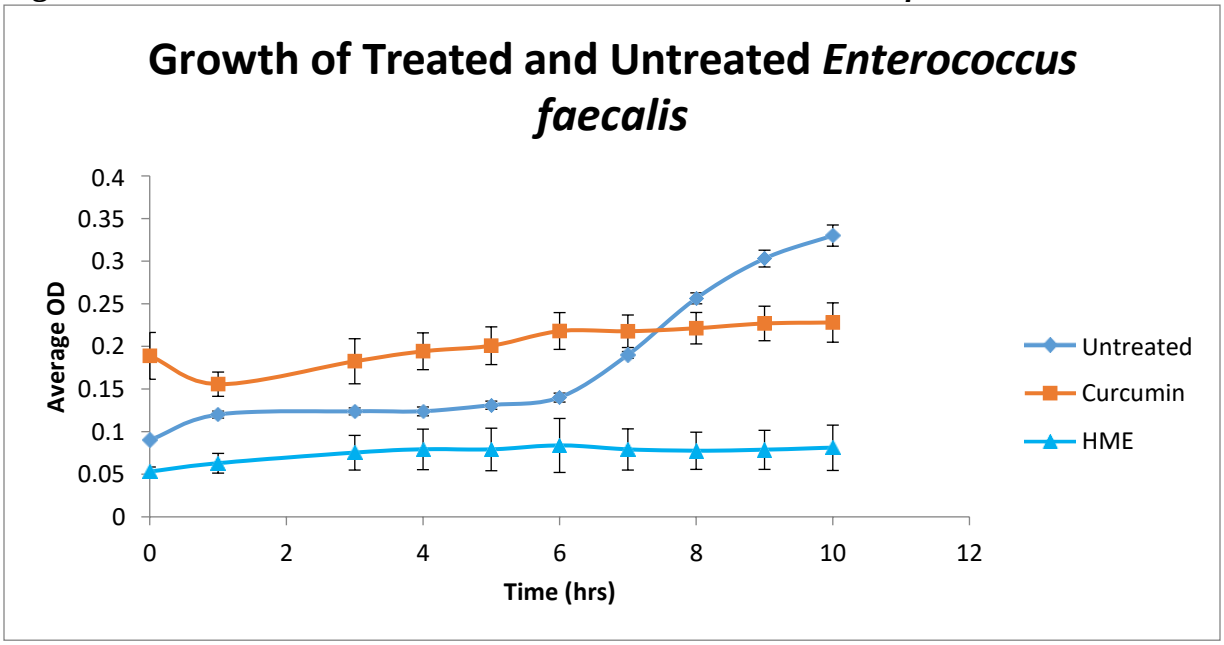

Figure 26. Average OD with standard deviation for untreated, curcumin treated, and HME treated *Enterococcus faecalis.*

c. *Escherichia coli*

Escherichia coli had a significant increase in OD over the ten hour period, with a starting OD of about 0.198 nm and increasing to about 0.8. *E. coli* treated with curcumin did have some increase in OD as well, but the increase was significantly less control (Figure 27). The increase in curcumin treated OD was about half the increase of untreated OD. *E. coli* treated with HME had no increase in OD, and instead saw a small decrease in

OD over time. The results indicated that curcumin did have some effect against *E. coli*, and that HME was able to completely inhibit the growth of the bacteria.

Figure 27: Growth Curve of Treated and Untreated *Escherichia coli*

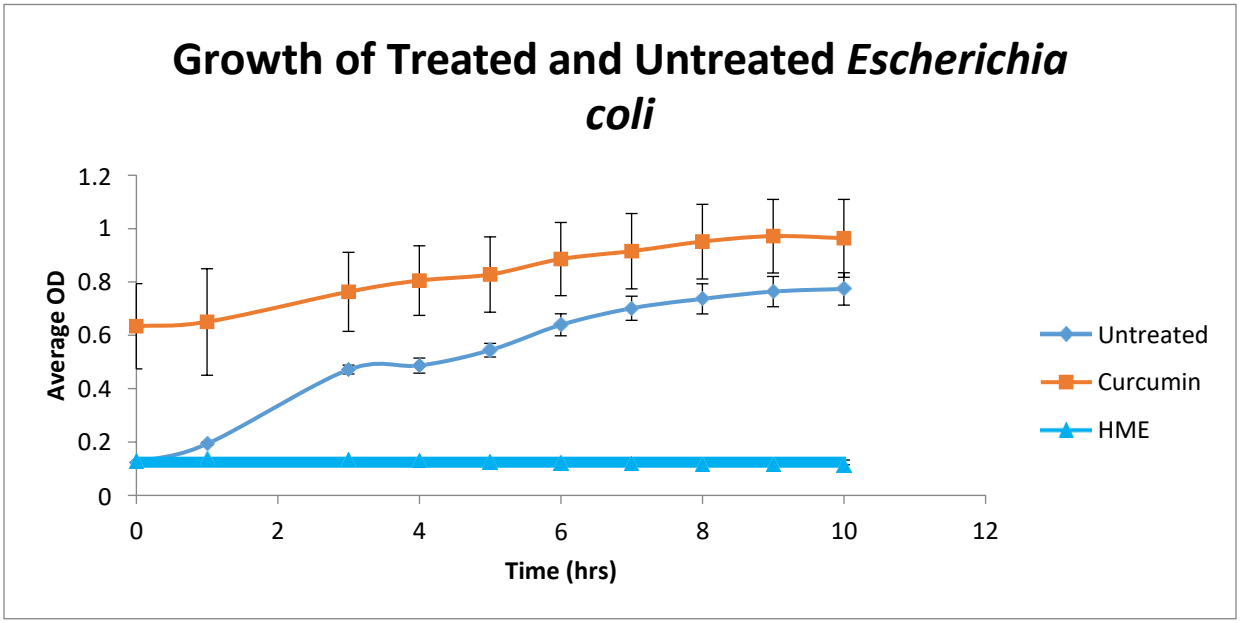

Figure 27. Average OD with standard deviation for untreated, curcumin treated, and HME treated *Escherichia coli*.

d. Pseudomonas aeruginosa

Pseudomonas aeruginosa had significantly increasing OD over the ten hour reading period when untreated (Figure 28). *P. aeruginosa* treated with curcumin did have a small increase in OD, and it seemed that curcumin was unable to completely inhibit the growth of this bacteria, although it was less than the control. However, *P. aeruginosa* treated with HME had a decrease in OD overtime.

Figure 28: Growth Curve of Treated and Untreated *Pseudomonas aeruginosa*

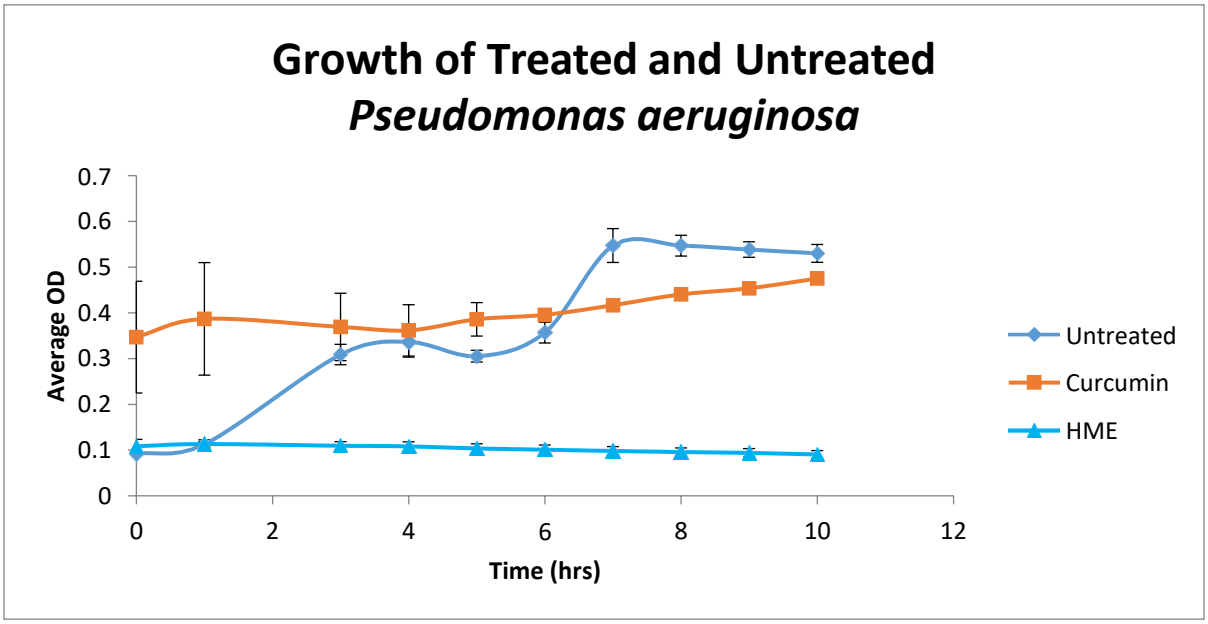

Figure 28. Average OD with standard deviation for untreated, curcumin treated, and HME treated *Pseudomonas aeruginosa.*

e. Mycobacterium smegmatis

Mycobacterium smegmatis had significant OD growth for both the untreated and curcumin treated bacteria, with both ODs increasing by about 0.5-0.6 (Figure 29). This was expected, since none of these experiments have found curcumin to be overly effective against *M. smegmatis*. In contrast, *M. smegmatis* treated with HME did not see an OD increase at all, and instead saw OD decrease over the ten hour period. This suggests that while curcumin is not effective against *M. smegmatis*, HME is significantly effective.

Figure 29: Growth Curve for Treated and Untreated *Mycobacterium smegmatis*

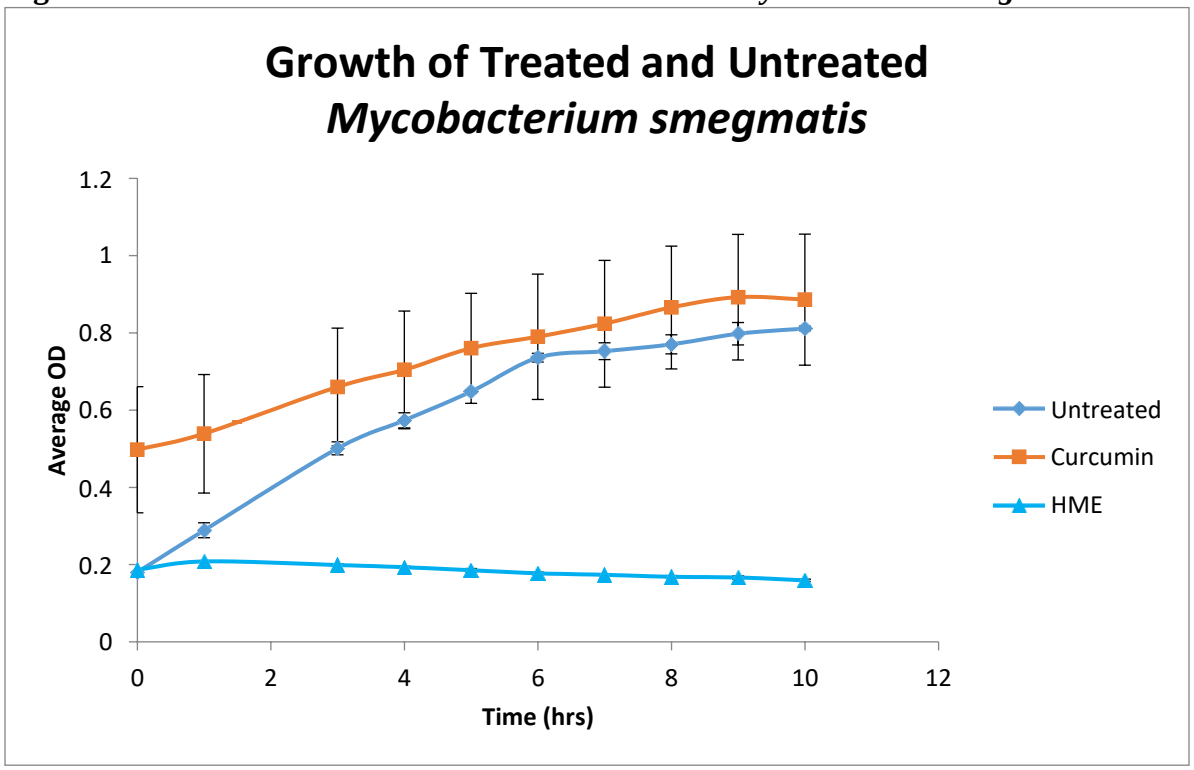

Figure 29. Average OD with standard deviation for untreated, curcumin treated, and HME treated *Mycobacterium smegmatis.*

Overall, both compounds do inhibit bacterial growth. HME inhibited the growth of all bacteria, including *M. smegmatis*. Curcumin was unable to stop *M. smegmatis* growth, but it did significantly stop *S. aureus* and *E. faecalis*. The growth of *E. coli* and *P. aeruginosa* was still less than the growth of the controls, but more than that of the gram positive curcumin treated bacteria. This experiment suggests that curcumin works better on gram positive bacteria than on gram negative bacteria, while HME is indiscriminate.

Determining the Effect of Curcumin and HME on Biofilms

a. Congo Red Assay

After curcumin and HME were determined to be very effective against most of the bacteria used in this study, both compounds were tested against biofilms formed by *S. aureus, E. faecalis, P. aeruginosa, E. coli,* and *M. smegmatis* in several ways. A 24 well Congo Red plate was

utilized for one portion of the experiment. Four wells were used for each of the five microorganisms: a negative control, the untreated microorganism, the microorganism treated with 50 µM curcumin, and the microorganism treated with 50 µM HME. Plates were incubated and checked every 24 hours for 72 hours (Figure 30). The results after the full 72 hours suggest that curcumin does have some effectiveness against biofilm formation, but it does not stop bacterial growth completely. However, bacteria treated with curcumin did not form full biofilms like untreated bacteria did. The results regarding HME for this test were less conclusive, and based on the results of this experiment it cannot be concluded with certainty that HME plays a role in stopping biofilm growth. This suggests further studies need to be carried out to determine whether or not HME is successful against biofilms.

Figure 30: Congo Red Biofilm Evaluation of *S. aureus, E. faecalis, P. aeruginosa, E. coli,* and *M. smegmatis* Untreated, Treated with Curcumin, and Treated with HME

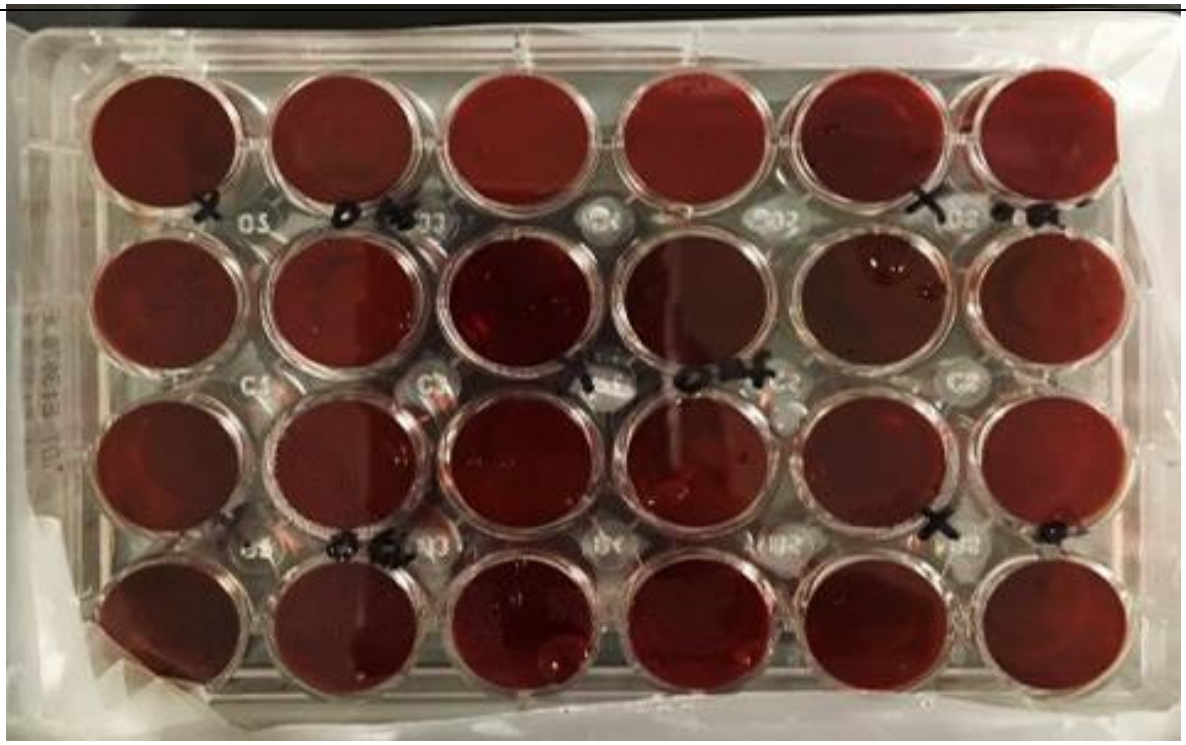

Figure 30. Biofilm assessment on a Congo Red plate after 72 hours for *S. aureus* (1-4), *M. smegmatis* (5-8), *E. faecalis* (9-12), *E. coli* (13-16), and *P. aeruginosa* (17-20).

b. Crystal Violet Assay

A crystal violet (CV) assay was also done to evaluate the effectiveness of 50 µM curcumin and HME treatments on biofilms. The results of this assay were slightly more conclusive than the Congo Red assay (Figure 31). However, % inhibition values (Table 4) were not overly impressive for either curcumin or HME. Crystal violet was allowed to soak in each well for a half hour before the stain was removed and the 48 well plate was left at room temperature for 24 hours. Biofilm growth was measured in OD (nm) after 1 ml of acetic acid was added to each well of the 48 well plate. If the compound was successful in biofilm inhibition, the OD would be higher for the untreated biofilm than for the treated biofilms. For this assay, curcumin was shown to inhibit biofilm formation in *S. aureus, E. faecalis,* and *E. coli*. It did not appear to inhibit biofilm formation in *P. aeruginosa* and *M. smegmatis.* HME worked well in inhibiting biofilm formation in *S. aureus, M. smegmatis, E. coli,* and *P. aeruginosa,* but not *E. faecalis.* Overall, % inhibition values (Table 4) are higher for HME than for curcumin. For example, *S. aureus* had values of 55% inhibition for curcumin and 85% inhibition for HME. Values for *E. coli* were about the same (roughly 39-40%) for both compounds. For *M. smegmatis*, there was no inhibition for curcumin and about 33% inhibition for HME. These results are mostly consistent with previous results in this study for both compounds, except for the failure of curcumin to inhibit the *P. aeruginosa* biofilm. Further studies are needed to reach definitive conclusions on the effects of these compounds against biofilms.

Figure 31: Measuring the Effectiveness of Curcumin and HME on Biofilms through a Crystal Violet Assay

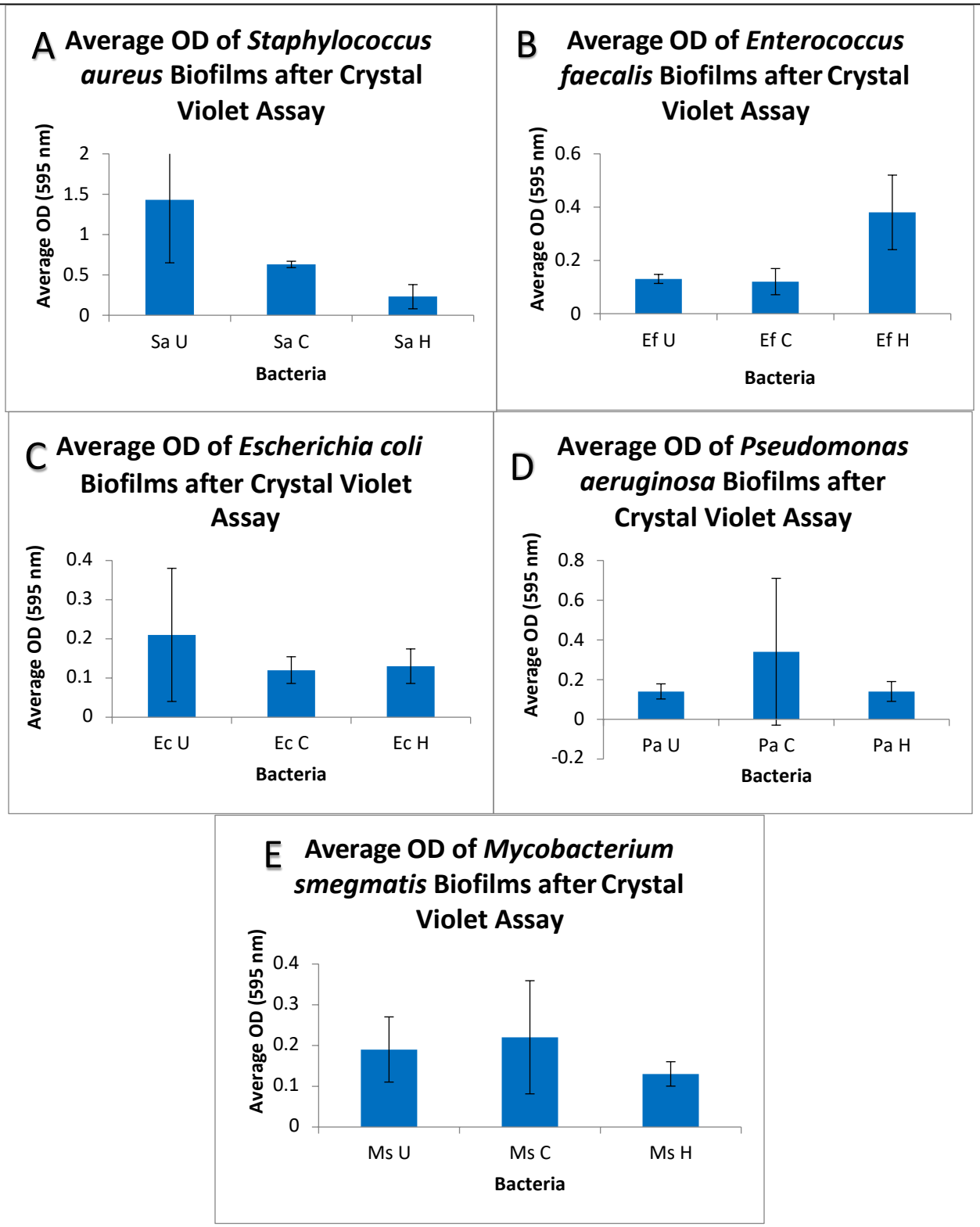

Figure 31. Crystal Violet Assay shows average optical density (OD) of biolfims formed by *Staphylococcus aureus* (A), *Enterococcus faecalis* (B), *Escherichia coli* (C), *Pseudomonas aeruginosa* (D), and *Mycobacterium smegmatis* untreated, treated with 50 µM of curcumin and treated with 50 µM of HME.

Table 4. % Inhibition Values for Crystal Violet Assay

Bacteria	C % Inhibition	HME % Inhibition
Sa	55	85
Ef	7.4	0
Ec	40.1	39
Pa	0	6.2
Ms	0	33

Table 4 shows % inhibition values from the CV assay for curcumin and HME.

c. Resazurin Assay

A resazurin assay was also done to determine if 50 µM curcumin and HME treatments are effective against biofilms. This assay also involved the use of a 48 well plate. About 24 hours before the reading was taken, 1X PBS and 20 µM resazurin were added to each well. Fluorescence was measured through reflective light units (RLUs) at 560 nm excitation and 590 nm emission. Of the three biofilm assays conducted, the results of the resazurin assay were the most promising. If the compound was successful in biofilm inhibition, the RLUs would be higher for the untreated biofilm than for the treated biofilms. This assay showed curcumin being effective against *S. aureus*, *E. faecalis*, *E. coli*, and *P. aeruginosa* biofilms, but not against *M. smegmatis* biofilms. HME reduced biofilm growth in all five microorganisms in comparison to the untreated biofilms (Figure 32). However, % inhibition values (Table 5) are still significantly lower than % inhibition values for the CFU assays. For this assay, *E. faecalis* biofilms were inhibited by both compounds, but % inhibition values were around 51% for curcumin compared to only 14% for HME. Values for *E. coli* and *P. aeruginosa* were similar. The results for the three different biofilm assays were not overly conclusive, and poor inhibition suggests biofilms are harder to treat than individual CFUs.

Figure 32: Measuring the Effectiveness of Curcumin and HME on Biofilms through a Resazurin Assay

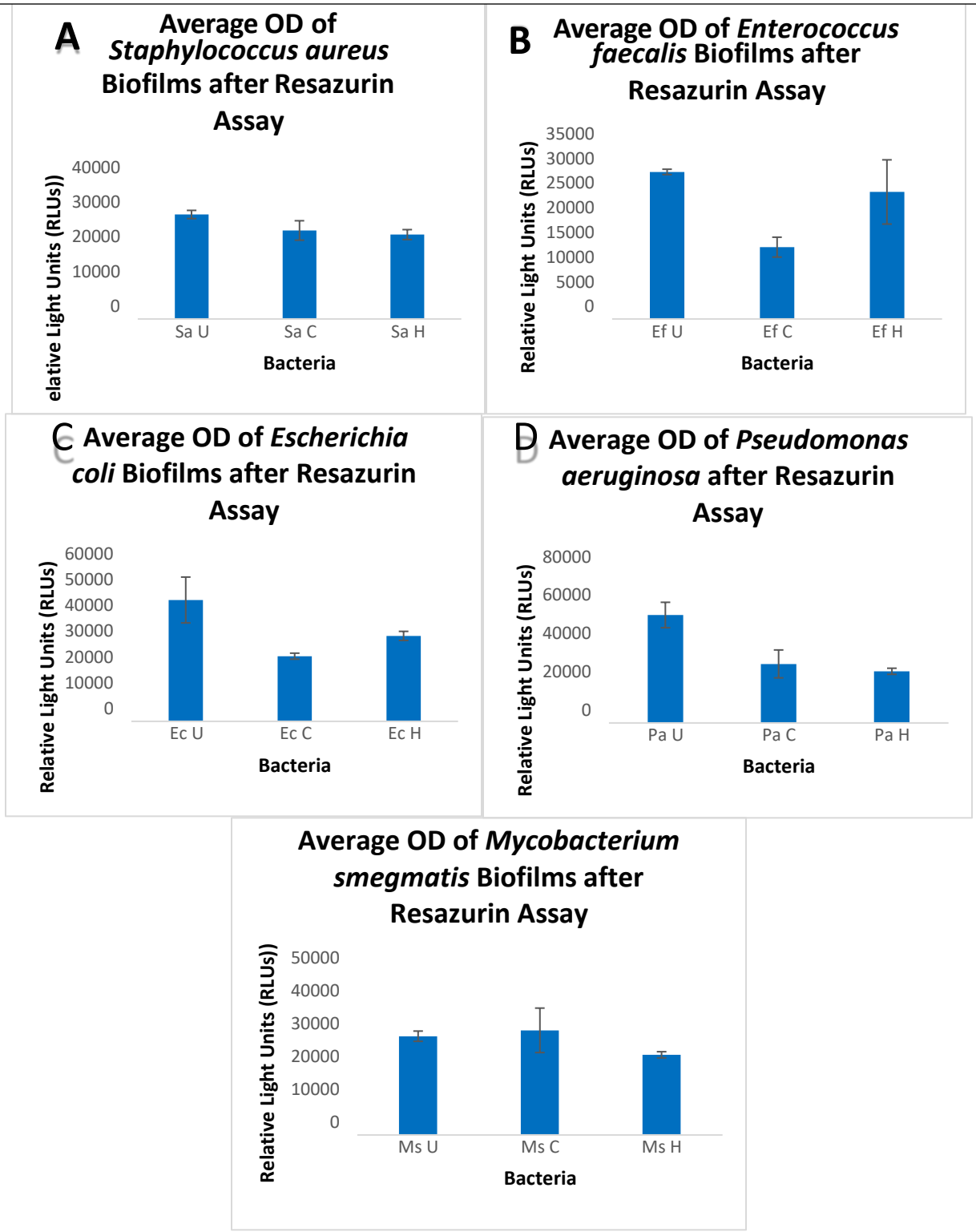

Figure 32. Fluorescence was measured at an excitation of 560 nm and an emission of 590 nm for *Staphylococcus aureus* (A), *Enterococcus faecalis* (B), *Escherichia coli* (C), *Pseudomonas aeruginosa* (D), and *Mycobacterium smegmatis* (E) biofilms that were untreated, treated with 50 µM curcumin, and 50 µM HME.

Table 5. % Inhibition Values for Resazurin Assay

Bacteria	C % Inhibition	HME % Inhibition
Sa	15.3	19.3
Ef	51.2	14
Ec	44	30
Pa	45	52
Ms	0	19

Table 5 shows % inhibition values from the Resazurin assay for curcumin and HME.

Overall the CV and Resazurin assay showed more promise than the Congo Red Assay. Due to the unimpressive results for all three, however, it is clear that biofilms are difficult to treat and that higher concentrations of the compounds may be required. These experiments should be repeated with higher concentrations.

Live-Dead Assay Through Fluorescent Microscopy

Live-Dead assays were done on each of the five microorganisms to compare the number of live and dead cells in the control, curcumin treated, and HME treated samples. Stock plates were prepared for each of the five bacteria and incubated overnight at 37°C. Tubes were prepared for two hour treatments of both 50 µM curcumin and 50 µM HME. Slides were prepared with a loopful of bacteria and 5 µl of a 60 Green: 40 Red Live-Dead dye and viewed under the fluorescent microscope at 40X.

a. Staphylococcus aureus

All slides were viewed under the fluorescent microscope at 40X. The control slide for *S. aureus* showed mostly green, or living, cells, as would be expected in the control (Figure 33). Treated slides for curcumin and HME suggested the treatments were successful, as the cells were

mostly red compared to the control's mostly green cells. This assay shows that both compounds resulted in heavy death of *S. aureus*.

Figure 33: Live-Dead Assay for *Staphylococcus aureus*

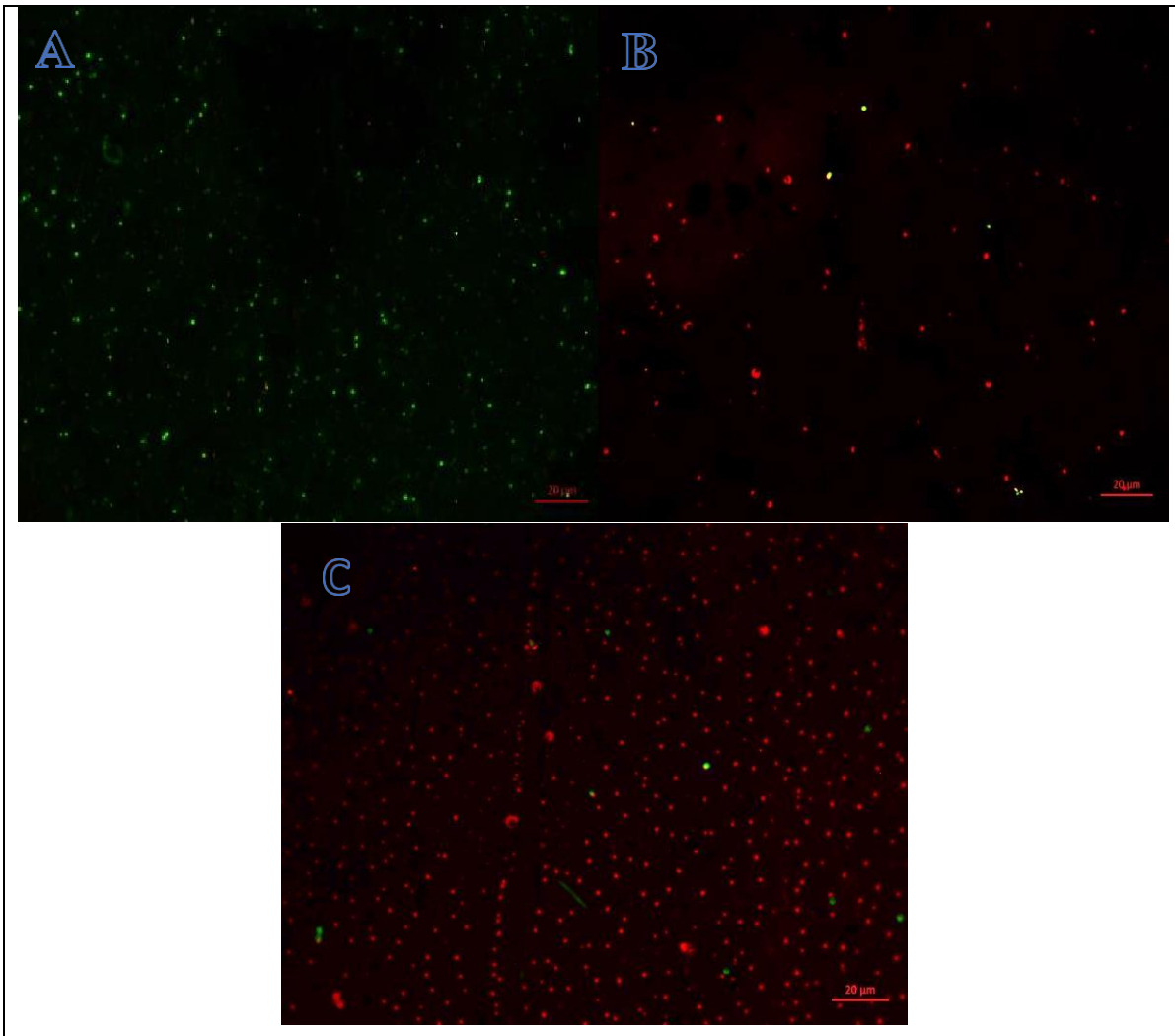

Figure 33. Live-Dead Assay of *Staphylococcus aureus* untreated (A), treated with curcumin (B), and treated with HME (C).

b. Enterococcus faecalis

All slides were viewed under the fluorescent microscope at 40X. The control slide for *E. faecalis* showed mostly green cells, suggesting that most of the cells were alive, as they were

expected to be (Figure 34). Treated slides for curcumin and HME suggested the treatments were successful, as the cells were mostly red compared to the control's mostly green cells. This assay shows that both compounds resulted in significant death of *E. faecalis*.

Figure 34: Live-Dead Assay for *Enterococcus faecalis*

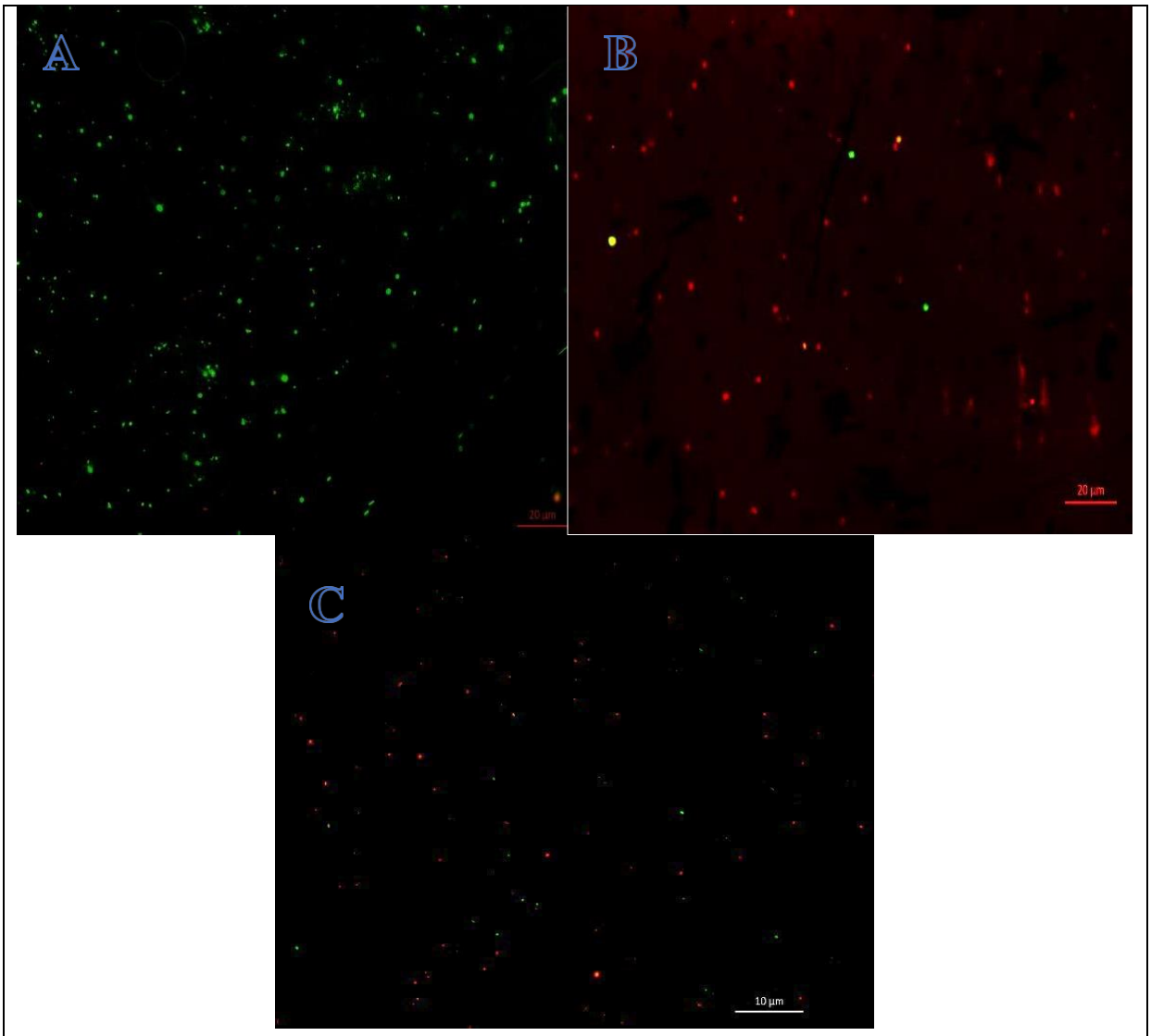

Figure 34. Live-Dead Assay of *Enterococcus faecalis* untreated (A), treated with curcumin (B), and treated with HME (C).

c. Escherichia coli

All slides were viewed under the fluorescent microscope at 40X. The control for *E. coli* showed most green cells, which was expected. Both curcumin and HME treated *E. coli* resulted in mostly red cells, and there were very few green cells, especially compared to the untreated

sample (Figure 35). These results suggest that both compounds have at least some effectivity against *E. coli*.

Figure 35: Live-Dead Assay of *Escherichia coli*

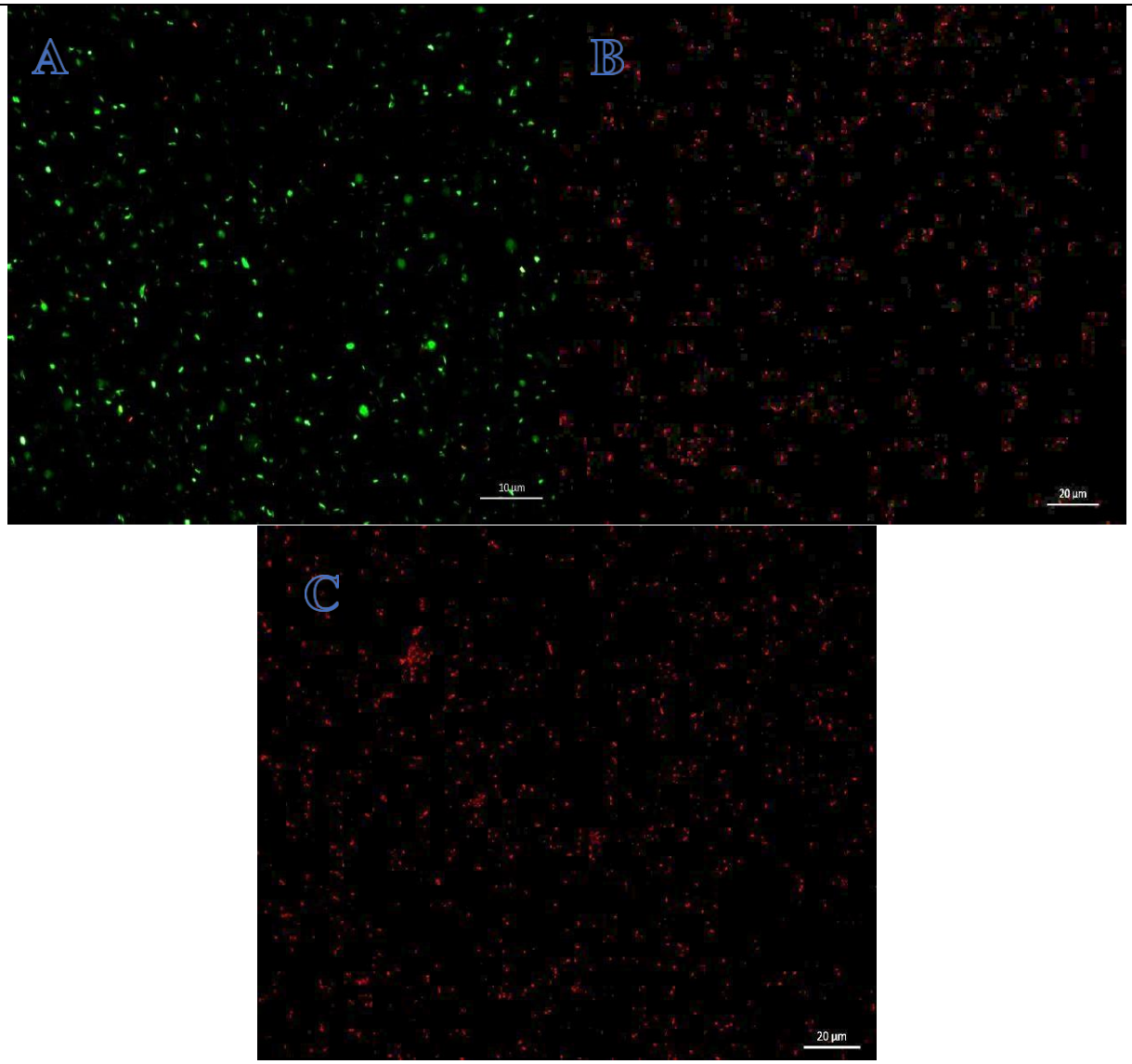

Figure 35. Live-Dead Assay of *Escherichia coli* untreated (A), treated with curcumin (B), and treated with HME (C).

d. Pseudomonas aeruginosa

All slides were viewed under the fluorescent microscope at 40X. The control for *P. aeruginosa*, like the other controls, showed mostly green cells. Both curcumin treated and HME treated *P. aeruginosa* showed mostly red cells under the microscope (Figure 36). This shows that

both compounds are at least moderately effective against *P. aeruginosa*, compared to how many living cells were present in the untreated sample.

Figure 36: Live-Dead Assay of *Pseudomonas aeruginosa*

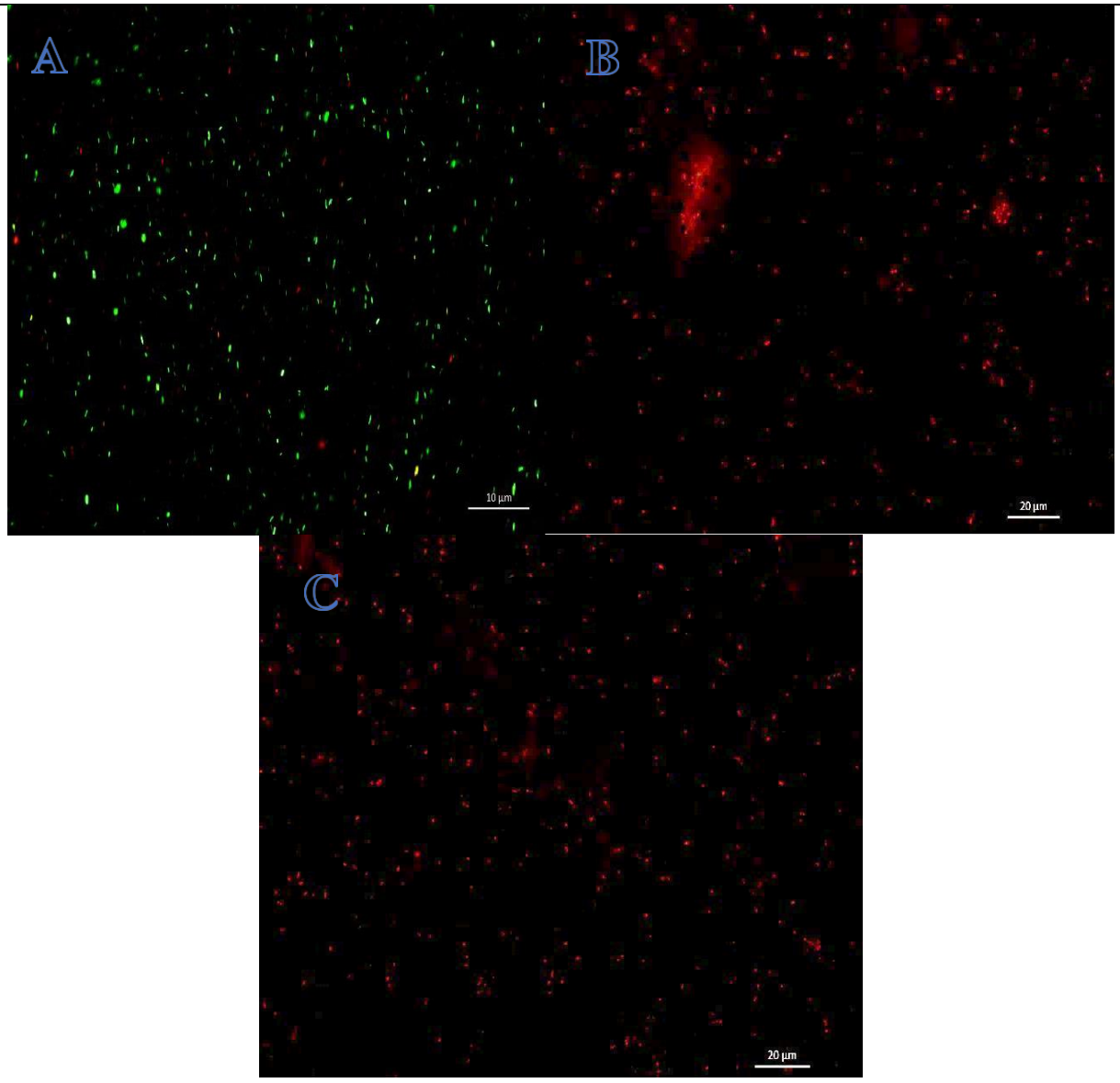

Figure 36. Live-Dead Assay of *Pseudomonas aeruginosa* untreated (A), treated with curcumin (B), and treated with HME (C).

e. Mycobacterium smegmatis

All slides were viewed under the fluorescent microscope at 40X. The control for *M. smegmatis* showed mostly green cells. Consistent with results from previous experiments, the curcumin treated *M. smegmatis* did still have a significant number of green cells, especially compared to

the HME treated *M. smegmatis*, where the cells were almost all red (Figure 37). This shows that HME works much better on *M. smegmatis* than curcumin, which does not work well on this specific bacterium at all.

Figure 37: Live-Dead Assay of *Mycobacterium smegmatis*

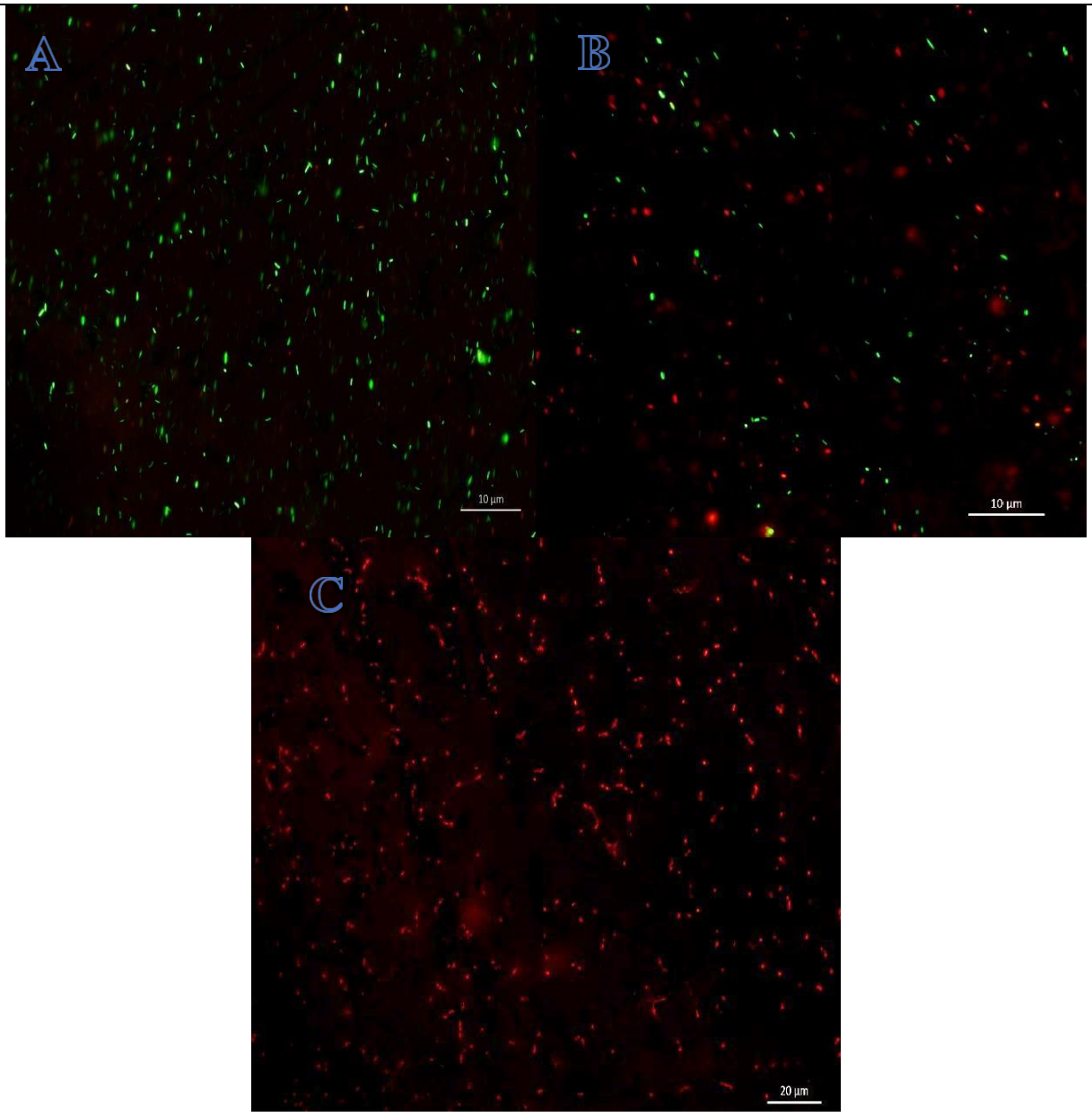

Figure 37. Live-Dead Assay of *Mycobacterium smegmatis* untreated (A), treated with curcumin (B), and treated with HME (C).

The Live-Dead assays correlated with the results of the previous experiments, showing HME treatment to be effective against all five of the microorganisms and curcumin treatment to be effective against all of the microorganisms but *M. smegmatis*. This assay confirms that treatment with these compounds do result in bacterial cell death.

4. Conclusions

Curcumin

The results of this study confirmed that curcumin is effective against not only *S. aureus,* but against *E. faecalis, E. coli,* and *P. aeruginosa* as well. The CFU assays showed the effectiveness of curcumin against *S. aureus, E. faecalis, E. coli,* and *P. aeruginosa,* but not against *M. smegmatis*. It can be concluded from these results that curcumin is effective against both gram positive and gram negative bacteria, but not against acid-fast bacteria. Overall, the % inhibition values were higher for gram positive bacteria treated with curcumin than for gram negative bacteria treated with curcumin. Gram negative bacteria are more likely to exhibit resistance to antibacterial compounds due to an extra cell membrane that gram positive bacteria do not possess (Zgurskaya *et al.*, 2016). However, all % inhibition calculations showed significant inhibition in all microorganisms treated with curcumin besides *M. smegmatis*.

The growth curve confirmed curcumin to be somewhat effective against all of the bacteria used in this study except for *M. smegmatis*. OD values decreased for curcumin treated *S. aureus* and *E. faecalis,* while they increased slightly for *E. coli* and *P. aeruginosa*. For *M. smegmatis* growth occurred at a similar rate for treated and untreated bacteria. Curcumin's effectiveness was also

shown by the Live-Dead assays done for each microorganism, which showed mostly green, living cells for the control bacteria and mostly red, dead cells for the curcumin treated bacteria.

Results for the Congo Red assay suggest that curcumin has some effectiveness against biofilms, but that it does not stop biofilm growth completely. This is corroborated by data collected from the Crystal Violet (CV) assay. The Resazurin assay offered more conclusive results for the potential effect of curcumin on biofilms. These assays both showed that while curcumin did have some effect against the biofilms formed by the microorganisms besides *M. smegmatis*, it did not produce overwhelming % inhibition like it did in the CFU assays. This study therefore indicated that biofilms are harder to treat than individual CFUs are, and in future experiments, the concentration used to treat biofilms may need to be higher than 50 µM. Biofilms are known to be harder to treat than singular bacterial colonies, and they are more likely to be antibiotic resistant as well (Hughes & Webber, 2017). The results in this study regarding curcumin's antibacterial effects correlate with results from previous studies regarding the antibacterial effects of curcumin when used on various types of gram negative and gram positive bacteria. The results also suggest that curcumin is most effective on gram positive bacteria, moderately effective on gram negative bacteria, and not effective on acid-fast bacteria.

HME

Currently, whether or not hispolon will be a widely effective antibacterial agent is not yet well known, although the results of this study were promising. The results of the CFU assays suggest that HME is at least somewhat effective against the growth of all five microorganisms, including *M. smegmatis*. Compared to curcumin, however, % inhibition values were slightly lower for all bacteria besides *S. aureus* and *M. smegmatis*. Unlike with curcumin, HME does not seem to work best specifically on one type of cell wall compared to others.

In addition, the growth curve confirmed HME to be at least somewhat effective against all five bacteria used in the study. For all HME treated bacteria, OD values decreased throughout the 10 hour reading period, compared to the increase in OD values in all of the untreated bacteria. HME works especially well against *M. smegmatis* compared to curcumin. HME's effectiveness was also shown by the Live-Dead assays done for each microorganism, which showed mostly green, living cells for the control bacteria and mostly red, dead cells for the HME treated bacteria. However, much future research is needed to determine if in fact hispolon derivatives will ever be a viable option to treating bacterial infections.

Similarly to curcumin, HME did not seem to tremendously inhibit the biofilms based on calculated % inhibition values. The Resazurin assay offered the most promising results, but the biofilm assays were not as successful as the colony forming unit assays. This study indicated that biofilms are harder to treat than individual CFUs, and in future experiments, the concentration used to treat biofilms may need to be higher than 50 µM. As previously stated, biofilms are expected to be more difficult to treat (Hughes & Webber, 2017). Future experiments should also include testing hispolon and antibiotics together against biofilms to see if results are improved.

HP

Given the overall success of HME against the various bacteria used in this study, HP was tested as well to see if a second hispolon derivative would yield the same results. Like HME, HP has been shown to be effective against *Mycobacterium* (Balaji *et al.,* 2019). However, just from the results of the CFU assays for this compound, it is clear that HP does not have the same antibacterial effect against gram positive and negative bacteria that it does against acid-fast bacteria. The CFU graphs shows very little, if any inhibition, for any of the bacteria besides *M. smegmatis*. For *S. aureus, E. faecalis, P. aeruginosa,* and *E. coli,* the treated plates all have just

as much, if not more, growth as the control plates. *M. smegmatis* had % inhibition numbers of 85% or above for all three concentrations, while the % inhibition numbers for all other bacteria was 54% or below. In some cases, treated plates had more growth than the control plates, resulting in an inhibition value of zero. It is possible that while HME has the ability to prevent mycolic acid synthesis and affect the cell walls of gram negative and gram positive bacteria, HP may only prevent mycolic acid synthesis (Balaji *et al.,* 2016, Balaji *et al.,* 2019). Due to the unsuccessful CFU assays, other experiments were not completed for this compound as they were for HME and curcumin. This compound only appeared to work on *M. smegmatis*.

Overall Conclusions

In conclusion, curcumin and HME look to be effective antibacterial agents against various types of gram positive, gram negative, and in the case of HME, acid-fast bacteria. Based on the results of this study, curcumin appears to be most effective on gram positive bacteria than gram negative bacteria, which is often the case for various antibacterial treatments (Zgurskaya *et al.*, 2016). It does not seem to be effective against acid-fast bacteria. HME, however, appears to be effective against gram positive, gram negative, and acid-fast bacteria. It is overall more effective against the gram negative bacteria than curcumin was, and definitely more effective on acid-fast bacteria. HP, while effective on acid-fast bacteria, did not show much promise of being effective against gram negative or gram positive bacteria.

Future Studies

Some relevant future studies may include investigating more of the many hispolon derivatives as antibacterial agents, as well as exploring the effectiveness of both curcumin and hispolon derivatives in concert with antibiotics. Curcumin and HME should be tested at concentrations above 50 µM for biofilm activity, and once again, in synergy with antibiotics to determine if that

improves their effectiveness. Based on the results of this study, both curcumin and HME are promising as future antibacterial treatments.

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